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The Effects of Age and Induction on Cockroach Mixed Function Oxidase Activity and Cell Morphology

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THE EFFECTS OF AGE AND INDUCTION ON COCKROACH MIXED FUNCTION OXIDASE ACTIVITY AND CELL MORPHOLOGY

by

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Approved:

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ABSTRACT

The Effects of Age and Induction on Cockroach

Mixed Function Oxidase Activity and

Cell Morphology

by

Richard L. Turnquist, Doctor of Philosophy

Utah State University, 1972

Major Professor: Dr. William A. Brindley
Department: Zoology

Female American cockroaches showed definite age-dependent changes in levels of activity of the microsomal mixed function oxidase chain. Cytochrome P-450 levels, EPN-detoxication, and p-nitroanisole O-demethylation activities were very low in young adult insects but increased steadily reaching a peak at about 100 days in fat body and at about 90 days in midgut and hindgut. The activities then declined rapidly reaching levels of young insects at about 130 to 140 days of age. NADPH-NT-reductase activity was high in young insects and declined during the first few weeks of adult life. This activity too showed a peak at about 100 days.

Injections of chlorcyclizine, a known microsomal enzyme inducer, significantly increased levels of cytochrome P-450, EPN-detoxication, p-nitroanisole O-demethylation and NADPH-NT-reductase activities in young

cockroaches. The inductive injections were effective, however, only before the natural activity peak was reached at 100 days. Beyond this point the injections had no inductive effect indicating that the microsomal mixed function oxidase chain in this insect is uninducible when normal enzyme levels are falling.

NADPH-NT-reductase activity in male cockroaches, while being somewhat higher than in females, showed a similar age-dependent curve with the peak occurring at about 120 days.

Age-dependent carbaryl resistance in male and female insects tended to follow levels of the mixed function oxidase activities. Fifty to sixty day old insects however, tended to be more resistant to the insecticide than microsomal enzyme levels would indicate.

RNA levels of normal female insects showed age-dependent curves similar to those of the microsomal enzyme activities being low in young adults and reaching a peak at about 100 days. Chlorcyclizine injections had little or no effect on total microsomal RNA levels. Surprisingly, actinomycin D, an RNA synthesis inhibitor, increased the inductive effect of chlorcyclizine at all ages.

Electron microscopy indicated that as enzyme levels increased the morphology of the endoplasmic reticulum changed. Tissue with low enzyme levels contained rough lamellar reticulum which became irregular or tubular in form as enzyme levels increased and finally became typical smooth reticulum in tissue with very high enzymatic activities. When enzyme levels began to fall the endoplasmic reticulum largely disappeared and concentric membrane whorls

appeared in the cells. The whorls showed positive acid phosphatase activity. In older insects, which had low enzyme activities, rough lamellar endoplasmic reticulum was once again evident along with many large and very complex membrane whorls. The endoplasmic reticulum of induced tissue was characteristic of the enzyme levels attained in these insects. The ultrastructure of tissues with similar enzyme levels, whether normal or induced, tended to be very similar.

(107 pages)

INTRODUCTION

Within the past decade the phenomenon of enzyme induction has aroused a great deal of research interest. Induction plays an important role in drug therapy and xenobiotic detoxication. Although its mechanism is poorly understood the inductive process results in the increase of microsomal enzymes known as the "mixed function oxidases" (Mason, 1957). It is these enzymes which play an important role in the detoxication of foreign compounds.

Little is known about enzyme induction in insects and only a few recent reports have shown that the process even occurs in these animals (Balazs and Agosin, 1968; Ahmad and Brindley, 1971, Plapp and Casida, 1970). The process is however, potentially very important. It may play a role in insect pesticide resistance (Plapp and Casida, 1970) and perhaps could be used to protect beneficial insects from the effects of widespread pesticide usage.

Little work has been done in studying the effects of age on normal microsomal enzyme levels and almost none on its effect on enzyme induction. Often enzyme level changes produced by drug treatment are reported without regard to the age of the animal. If age does affect basal enzyme levels or an animal's response to the inductive treatment such reports could produce very misleading data.

An animal which showed age-dependent changes in basal levels of the mixed function oxidase enzymes could be used in studying the similarities and

differences between normal and induced enzyme level changes. This type of study might be helpful in understanding the mechanisms involved in enzyme induction and how basal enzyme levels affect the inductive process.

REVIEW OF LITERATURE

The mammalian liver was identified early in the 1950's as a most active organ for the enzymatic detoxication of foreign compounds (Axelrod, 1954). More specifically, it was established that it was a group of compounds called "mixed function oxidases" of the hepatocytes' endoplasmic reticulum (or its fragments, the microsomes) which were capable of converting xenobiotics into more polar and easily excretable compounds (Mason, 1957). An early review paper by Brodie, Gillette and La Du (1958) listed a series of reactions catalyzed by these enzymes. The list of mircorosmal oxidative reactions has grown considerably and now includes deaminations, O-dealkylations, alkyl and aromatic hydroxylations, epoxidations, N-dealkylations, N-oxide formations, N-oxidation of amines, S-demethylations, S-oxidations, phosphothionate oxidations, and dehalogenations (Gillette, 1966). All these reactions are characterized by an absolute requirement for NADPH and molecular oxygen.

The components of the mixed function oxidase electron transport chain have been determined during the past decade by researchers in several different laboratories. Klingenberg (1958) and Garfinkel (1958) independently reported the presence of a carbon monoxide-binding pigment in rat liver microsomes. Omura and Sato (1964) showed the pigment to be a hemoprotein and named it cytochrome P-450 due to its absorption spectrum when complexed in a reduced form with carbon monoxide. Williams and Kamin (1962) had previously isolated and

characterized a flavoprotein from liver microsomes which they called NADPH-cytochrome-c-reductase. This protein was capable of transferring electrons from NADPH to a wide variety of acceptors. Orrenius (1965a) showed both the flavoprotein and the pigment to be components of the oxidative drug-metabolizing enzyme system.

The mechanism for the hepatic oxidation of drugs utilizing a single enzymatic pathway is thought to involve the following steps. Oxidized cytochrome P-450 and the drug react to form a drug-cytochrome complex. This complex is then reduced by NADPH cytochrome-c-reductase either directly or indirectly through an unidentified carrier. The reduced cytochrome then combines with molecular oxygen to form an active complex which breaks down to give oxidized cytochrome P-450 and the oxidized drug (Davies, 1969).

Some workers feel that the rate limiting step in microsomal oxidations may be the reduction of the cytochrome-drug complex by the electron carriers (Lu, Strobel and Coon, 1970). Ichikawa, Yamano and Fujishim, (1969) and his coworkers found that various in vitro detoxication rates did not necessarily follow cytochrome P-450 levels but were dependent upon rates of electron transfer somewhere in the chain. They also showed that NADH could be substituted as an electron donor but that its Michaelis constant was much larger. There are marked species differences in the rate of reduction of cytochrome P-450 even though there are few differences in the NADPH cytochrome-c-reductase or the cytochrome P-450 themselves. This indicates that either the kinetic properties of cytochrome P-450 vary from one species to another or that an unidentified carrier mediates the transfer of electrons from the flavoprotein to the cytochrome (Davies et al.,

1969). The adrenal cortex, which utilizes an electron transport chain containing cytochrome P-450 to hydroxylate steroids, has a non-heme iron carrier located between the cytochrome and the flavoprotein. No evidence for such a compound has been found in drug metabolizing systems.

During the past decade a great deal of research has centered on the phenomenon of enzyme induction. Stated briefly, enzyme induction is the increase in levels of microsomal oxidative enzymes following the in vivo administration of one of a large group of widely differing compounds. Since microsomal oxidative enzymes are relatively non-specific the administration on one inductive compound will lead to the increased metabolism of a large number of other substrates. Enzyme induction was first reported by Conney and his coworkers (1959, 1960) and since then has been shown to be important in chronic drug therapy, pesticide resistance, and pollutant residue excretion. Excellent recent reviews on enzyme induction have been written by Conney (1967), Kuntzman (1969), and Kupfer (1970).

It was Orrenius (1965b) who first reported that pretreatment of rats with inducing agents causing increased drug metabolism was accompanied by an increase in the cytochrome P-450 levels and in the activity of NADPH cytochrome-c-reductase. He and his coworkers (1965a, 1965c) also showed that the inductive treatment caused increases in the amounts of rough and smooth surfaced endoplasmic reticulum. Increases in microsomal RNA, protein and phospholipid were also reported. These investigators noted that rough endoplasmic reticulum proliferated rapidly immediately after the inductive treatment but after a few hours smooth reticulum also began to increase.

Prolonged periods of injection resulted in tissue which was tightly packed with smooth reticular vesicles and which had a much elevated detoxication activity. Since puromycin, a protein synthesis inhibitor, abolished the inductive effect it was concluded that the inducing agent caused an increased synthesis of microsomal drug metabolizing enzymes dependent upon the production of new membranes. They postulated that the new enzymes were synthesized by the rough endoplasmic reticulum which, when becoming saturated with enzyme, lost their ribosomes and changed to smooth reticulum.

Other workers (Gillette, Kamin and Sasame, 1968; Arcasoy and Smuckler, 1969; Staubli, Hess and Weibel, 1969; Weibel et al., 1969) confirmed the work of the Swedish investigators and it was assumed that increased production of detoxifying enzymes satisfactorily accounted for the increased metabolism seen in induction. Recently however, this hypothesis has come under attack. Kuriyama et al. (1969) reported that while phenobarbital injections did increase the amount of microsomal enzymes, the increase was not due to increased synthesis but rather to decreased degradation of the detoxifying enzymes. They reported that the half life of microsomal protein was increased as much as two-fold after phenobarbital administration. Levin and Kuntzman (1969) found that administration of 3-methylcholanthrene increased the half life of cytochrome P-450 significantly over control animals. They too felt that induction was the result of decreased degradation.

Others however, disagree. Greim et al. (1970) reported that the half lives of normal and phenobarbital-induced cytochrome P-450 were the same and that, in early stages at least, the increase in cytochrome P-450 levels

was the result of increased synthesis. They concluded that induction might be the result of a slower increase in the rate of degradation of cytochrome P-450 relative to the increase in synthesis. Nebert and Gelboin (1970) on the other hand, reported that induced increases in microsomal activity follow more classical protein synthesis mechanisms with induction causing synthesis of specific messenger RNA which, in turn, is translated at the ribosome to produce increased amounts of microsomal protein. To further confuse the issue, Holtzman (1969) has indicated that the induced microsomal constituents turn over independently of each other with the protein increase due to an increased synthesis without any decrease in catabolism while the phospholipid fractions show a decrease in catabolism with little or no increase in synthesis. Finally, Louis-Ferdinand and Fuller (1970) reported that following phenobarbital administration hepatic microsomal ribonuclease activity decreased as much as 99 per cent. This suggested to the authors that induction was mediated through suppression of enzymes capable of degrading messenger RNA.

One reason for confusion regarding inductive mechanisms might be that there are two classes of inducing compounds (Long, 1969). The first class which includes phenobarbital is made up of a group of diverse drugs including barbiturates, phenothiazines, chlorinated insecticides and phenylhydantoins. The second class including 3-methylcholanthrene consists almost entirely of polycyclic hydrocarbons. The two classes of compounds may increase microsomal enzymes by different mechanisms. Thioacetamide will prevent induction by phenobarbital but not by 3-methylcholanthrene (Long, 1969). Levin and

Kuntzman (1969) showed that the half lives and spectral properties of cytochrome P-450 induced by phenobarbital and 3-methylcholanthrene differed from each other with the phenobarbital induced hemoprotein being similar to that of control animals. The mechanisms controlling the formation of endoplasmic reticulum and the increase in cytochrome P-450 may also be different. Triazole will inhibit the increase in levels of cytochrome P-450 with phenobarbital administration but does not inhibit the proliferation of smooth endoplasmic reticulum or the increase in levels of NADPH cytochrome-c-reductase (Raisfeld et al., 1970). How these different mechanisms might affect the results reported by different investigators has not been determined but they certainly could account for some of the seeming discrepancies found in the literature.

The literature on microsomal oxidases and inductive effects in insects is not nearly as extensive as that found for mammals. The presence of microsomal detoxifying enzymes in insects was first reported by Agosin et al. (1961) and Arias and Terriere (1962). Their presence has now been demonstrated by many workers but the protection they offer to the insect is somewhat uncertain. Tsukamoto Shrivastava and Casida (1968) showed that the fifth chromosome in the house fly was responsible for conferring both high carbamate resistance and high activity of NADPH dependent microsomal oxidases indicating the possibility of a direct relationship between the two. Plapp and Casida (1969) confirmed this report and showed that resistant strains of flies metabolize pesticides at greater rates than susceptible strains. However, Schonbrod et al. (1968) using fourteen strains of house flies, concluded there is no simple relationship between insecticide resistance and levels of microsomal oxidases. They do play a

significant role however. Casida (1970) showed that methylenedioxyphenyl compounds, which compete directly for cytochrome P-450 and thereby inhibit the action of the mixed function oxidase chain, drastically lower the ability of an insect to resist pesticides.

Enzyme induction in insects is potentially very important. With the widespread use of commercial pesticides many insects are exposed to sublethal doses which might confer increased resistance if insect mixed function oxidases are inducible (Plapp and Casida, 1970). However, early attempts to show inductive effects in insects were unsuccessful (Oppenoorth and Houx, 1968) and some workers (Chakraborty and Smith, 1967; Meksongsee, Yang and Guthrie, 1967) even showed increased susceptibility to pesticides after inducement. Other evidence however, indicates that at least in some insects mixed function oxidases are inducible and may confer increased resistance to pesticides (Ahmad and Brindley, 1969; Plapp and Casida, 1970). In addition, Balazs and Agosin (1968) reported that pretreatment with DDT in house flies induced the synthesis of rapidly labeled RNA (probably messenger) which had a higher template activity than RNA from normal insects. These authors concluded that the inductive role of DDT is mediated by its ability to stimulate messenger RNA production and therefore protein synthesis.

Although many different parameters which might affect enzyme induction have been studied, comparatively little work has been done on the effects of age, especially in insects. Soyka (1969) measured N-demethylation activity in rats of increasing age and found that there was about a 3-fold increase in activity

up to an age of about thirty days. Kato and Takanaka (1968) studied age effects in rats and found that young rats had higher basal activities of microsomal oxidases and that these activities were increased more markedly by phenbarbital administration than those of older animals.

One of the few indications of age effects on microsomal enzymes in insects was reported by Nakatsugawa and Dahm (1965). While studying parathion activation in the American cockroach, a reaction catalyzed by microsomal mixed functions oxidases, they found the fat body of adult insects had an increasing ability to activate the insecticide up to an age of about three months. This indicates that there might be endogenous changes in the levels of the mixed function oxidases in this insect. This could provide an excellent opportunity to study the changes of basal enzyme levels and how these normal changes are related to and effect enzyme induction in insects.

MATERIALS AND METHODS

Insect culture

American cockroach (Periplaneta americana L.) colonies were reared in galvanized steel tubs and maintained on a diet of Purina Lab Chow. Newly-emerged adults were removed and put in age-labeled plastic laboratory cages at scheduled intervals. All insects were provided with a constant supply of food and water in a rearing room at a temperature of 34-37C. Biochemical and cytological experiments were conducted on adult female and male insects 1-140 days after moulting from the last nymphal instar to the adult.

Chemicals

EPN (O-ethyl-O-p-nitrophenyl phenylphosphorothioate) and chlorcyclizine dihydrochloride (1-(4-chlorobenzhydryl)-4-methyl-piperazine) were donated by American Cyanamid and DuPont laboratories respectively. All other reagents were purchased from commercial chemical companies.

Drug pretreatment

Chlorcyclizine dihydrochloride was dissolved in insect Ringer's solution (Thomsen, 1952) at a rate of 25 mg/ml. Sublethal doses were identified after injecting adult cockroaches with various amounts of the drug and determining the mortality after 24 hours.

Sublethal inductive doses (60 mg/kg of cockroach tissue) of chlorcyclizine solution were injected between the abdominal sternites with a syringe and micro-applicator. Injections were made 24 hours prior to conducting biochemical and cytological experiments. Insects used for experimental controls were injected with 2.4 ul of insect Ringer's solution.

Actinomycin D (Sigma Chemical Company; St. Louis, Missouri) was dissolved in Ringer's solution and injected (2 mg/kg of cockroach tissue) one-half hour prior to injection of chlorcyclizine (Schwartz and Garofalo, 1967).

Dissections

All dissections were made in a cold table at 0-4C. The cockroaches were decapitated and the legs removed. The body was pinned in a wax layer in a Petri dish, ventral side up, and covered with cold phosphate buffer (0.1 M, pH 7.4). A ventral incision was made in the abdomen and the body wall was spread with pins. Fat body, midgut including the enteric ceacae, and hindgut including the Malpighian tubules were removed with a fine forceps. All tissue was immediately placed in cold phosphate buffer in two dram glass vials and homogenized within one hour. Tissues from four or five insects were pooled for fat body experiments; gut tissues from two or three insects were pooled in midgut and hindgut experiments.

Preparation of microsomes

Gut tissue was removed from the vials and placed in a cold glass homogenizer tube containing 2.75 ml of cold phosphate buffer (0.1 M, pH 7.4) per gut. The tube was placed in a beaker of crushed ice and the tissue was

homogenized with 15 up-and-down strokes using a precooled power driven Teflon^{R} pestle. The homogenate was transferred to a seven ml centrifuge tube and spun at an average of 6,800 g for 10 minutes at 0 C in a Sorvall RC-2 centrifuge. The supernatant was transferred to another pre-cooled centrifuge tube and spun at an average of 20,000 g for ten minutes (Schonbrod and Terriere, 1966). The supernatant from this run was used in all midgut and hindgut biochemical experiments.

Fat body homogenates were prepared in the same way except that an additional centrifugation at 37,000 g for two hours (Schonbrod and Terriere, 1966) was used to bring down the microsomal tissue and separate it from the lipid of the fat body. The microsomal pellet was resuspended in phosphate buffer (10 mg/ml) for fat body biochemical experiments.

Cytochrome P-450 measurement

Cytochrome P-450 concentrations were determined according to the procedure of Sunderman (1968) with some modification. Two milliliters of the resuspended fat body microsomal solution were placed in each of two quartz one centimeter path length cuvettes. Two milligrams of sodium dithionite ($Na_2S_2O_4$) (J. T. Baker; Phillipsburg, New Jersey) were added to each. Oxygen-free nitrogen was bubbled through the reference cuvette and oxygen-free carbon monoxide was bubbled through the sample cuvette for two minutes. Samples were measured with a Zeiss PMQ-II spectrophotometer at 450 and 490 nm. The concentration of cytochrome P-450 was expressed as the optical density

difference between 450 and 490 nm per milligram of microsomal protein and per milligrams of wet microsomal tissue.

Measurement of NADPH-neotetrazolium-reductase activity

NADPH-NT-reductase activity was measured according to the procedure of Williams and Kamin (1962) in resuspended fat body microsomal solutions and in mitochondrial supernatant of midgut and hindgut homogenates. The microsomal source (0.5 ml) was added to an incubation mixture containing 0.4 ml of phosphate buffer (0.1 M, pH 8.0), 0.3 ml of neotetrazolium chloride (Nutritional Biochemicals; Cleveland, Ohio) solution (0.4 mg/ml water), and 0.3 ml of NADPH (Sigma Chemical Co.; St. Louis, Missouri) (4.6 mg/ml water). The mixture was aerobically incubated for 10 minutes at 37 C. The incubation was stopped by addition of 2.9 ml of a mixture of 40 ml of water, 3.6 ml of 10 per cent aqueous Triton X-100, 5.0 ml of 40 per cent formalin, and 10 ml of 1 M formate buffer (pH 3.5). The blank contained 0.5 ml of glass distilled water instead of the enzyme source. The production of reduced neotetrazolium was measured by reading at 555 nm on a Zeiss PMQ-11 spectrophotometer. Concentration was expressed as moles per milligram of microsomal protein or moles per milligram of wet microsomal tissue using an extinction coefficient of 2×10^6 cm^2/mole (Williams and Kamin, 1962).

Measurement of p-nitroanisole O-demethylation and EPN detoxication activity

Mitochondrial supernatant of midgut and hindgut homogenates and resuspended microsomes of fat body homogenates from normal and chlorcyclizine-induced cockroaches were used for EPN detoxication and p-nitroanisole O-demethylation activity studies. Activities were measured according to the procedure of Kinoshita, Frawley and DuBois, (1966) with some modification. Seven milligrams of EPN or p-nitroanisole were dissolved per milliliter of a mixture of 20 per cent ethanol and 80 per cent propylene glycol. Immediately before incubation, 0.5 ml of the EPN or p-nitroanisole stock solution was mixed with 5 ml of phosphate buffer (0.1 M, pH 7.8). The incubation mixture contained 0.2 ml of the EPN- or p-nitroanisole-buffer mixture, 0.1 ml NADPH (1 mg/ml water), and 0.8 ml of the microsomal suspension. The mixture was incubated for 60 minutes at 37 C. Controls used 0.8 ml of phosphate buffer instead of the tissue homogenates. The incubation was stopped by adding 1.8 ml cold acetone and 0.8 ml of glycine sodium hydroxide buffer (0.5 M, pH 9.5). The mixture was centrifuged at 10,000 g for 20 minutes. The amount of p-nitrophenol produced was measured at 410 nm with a Zeiss PMQ-II spectrophotometer. A standard curve was made by dissolving known amounts of p-nitrophenol in 1.2 ml of the incubation mixture. Activity was expressed as mg of p-nitrophenol produced per hour per milligram of protein.

RNA determinations

RNA determinations were made according to the procedure of Webb

(1956). One milliliter of the microsomal solution was added to 2 ml of 5 per cent trichloroacetic acid (aqueous) in a screw-top test tube and placed in a boiling water bath for 30 minutes. The solution was cooled and 2 ml of additional trichloroacetic acid were added. One milliliter of the acid-homogenate mixture was added to 1 ml of 6 N HCl and 1 ml of xylene (Mallinckrodt; St. Louis, Missouri) in a screw top test tube. The mixture was saturated with salt (NaCl), sealed and placed in a boiling water bath for 3 hours. The solution was cooled, 2 ml of xylene were added, and the mixture was centrifuged at 10,000 g for 20 minutes. Two milliliters of the xylene layer were added to 2 ml of a p-bromophenylhydrazine (J. T. Baker, Phillipsburg, Pennsylvania) solution (2.5 per cent in a solution of 2 ml of 37 per cent HCl and 100 ml of 95 per cent ethanol) in a 5 ml volumetric flask and placed in a 37 C bath for one hour. The solution was diluted to 5 ml with the acid-alcohol solution. A portion of the solution was transferred to a 1 cm path length quartz cuvette and the optical density measured in a Zeiss PMQ-II spectrophotometer at 450 nm. The blank contained 1 ml of trichloroacetic acid instead of the homogenate. A standard curve was made by dissolving known amounts of RNA in trichloroacetic acid. RNA concentration was expressed as mg RNA per mg of microsomal protein.

Protein determination

Protein determinations were made according to the method of Lowry et al. (1951). Three stock solutions were made prior to the determinations. Reagent A contained two grams of sodium carbonate per 100 ml of 0.1 N

sodium hydroxide. Reagent B was prepared by dissolving one gram of copper sulfate ($CuSo_4 \cdot 5H_20$) in 200 ml of a 1 per cent potassium tartarate solution. Commercial Folin-Ciocalteau phenol reagent was diluted two-fold with glass distilled water.

Immediately before protein determinations were made, 9.8 ml of reagent B were mixed with 0.2 ml of reagent A. One milliliter of the solution was mixed with 0.2 ml of the microsomal source and allowed to stand at room temperature for 10 minutes. One-tenth of a milliliter of the phenol reagent was added and the tubes were shaken vigorously and allowed to stand at room temperature for 30 minutes. The solutions were read at 550 nm on a Zeiss PMQ-II spectrophotometer with the blank containing 0.2 ml of distilled water instead of the microsomal source. A standard curve was prepared by dissolving known amounts of bovine serum albumin in the same phosphate buffer used to make the microsomal homogenates. Protein was expressed as mg/ml of solution.

Cytological techniques

Midgut and fat body were used in cytological studies. All dissections were made with the insects immersed in the fixation medium.

Tissue removed from the insects was diced into 0.5-1.0 nm blocks and immediately placed in Karnovsky's fixative (Karnovsky, 1965) in 0.2 M cacodylate buffer (pH 7.4). After fixation for two hours at room temperature, the tissue blocks were rinsed twice in cacodylate buffer and postfixed for 1-2 hours in 2 per cent osmium tetroxide (OsO_4). The tissue was again rinsed

twice in buffer. The tissue was dehydrated in increasing concentrations of alcohol according to the following schedule; seven minutes in 35 per cent, 15 minutes in 50 per cent, 15 minutes in 70 per cent, 15 minutes in 95 per cent, and three changes of two minutes each in absolute alcohol. The last change of alcohol was removed and the tissue was passed through three changes of five minutes each of cold propylene oxide. The third change of propylene oxide was poured off and one milliliter of fresh propylene oxide was added.

While the tissue was being dehydrated, a mixture of Epon 812 was prepared. Two stock solutions, one containing 67 ml of Epon 812 and 100 ml of dodecenyl succinic anhydride, and the other containing 100 ml of Epon 812 and 81 ml of nadic methyl anhydride, were prepared beforehand. Four parts of the first solution were mixed thoroughly with six parts of the second. A volume of 2,4,6-dimethylaminomethyl phenol equal to 1.5 per cent of the total mixture was added. After mixing, 1 ml of the Epon solution was added to the propylene oxide containing the tissue and the contents were mixed by gentle swirling. After one hour another two milliliters of the Epon was added and the mixture was allowed to stand at room temperature overnight. BEEM^{R} embedding capsules were filled within two millimeters of the top with the Epon mixture and the tissue was transferred into them with a wooden dowel. The Epon was hardened by three 12 hour polymerizations in ovens of 35, 45, and 60 C (Luft, 1961).

Sectioning was done on a Porter Blum II ultramicrotome with a DuPont diamond knife. Sections were floated on water and picked up with 75-300 mesh

copper grids. Sections were stained for 10 minutes in uranyl acetate and five minutes in lead citrate (Reynolds, 1963) and examined with a Zeiss EM-9A electron microscope.

Tissue for acid phsophatase study was fixed for two hours in cold 6.25 per cent glutaraldehyde buffered to pH 7.2 with 0.2 M tris-maleate buffer (pH 5.2) (Squier et al., 1970). The blocks were washed overnight in the same buffer. Ten milliliters of 0.05 M tris-malaeate buffer (pH 5.2) were warmed to 37 C and five milliliters of 0,1 M aqueous sodium betaglycerophosphate (pH 5.2 with HC1) were added. Immediately before staining, 10 ml of 0.006 M aqueous lead nitrate were added by drops to the solution. The tissue was incubated in this mixture for 60 minutes at 37 C and then washed in buffer for five minutes. The blocks were fixed in 2 per cent OsO_4 for two hours and dehydrated, embedded, and stained as before.

Carbaryl toxicity

Insects of 80 days of age were given topical applications of carbaryl dissolved in acetone (50 µg/µl) to determine the approximate LD_{50} dose. A value of 150 µg/g was obtained for females and 100 µg/g for males. This dosage was applied topically with a syringe and microapplicator to insects of 18, 37, 55, 80, 102 and 130 days of age. Mortality was determined 24 hours after application.

RESULTS

Figures 1 through 10 indicate that microsomal mixed function oxidase activities in American cockroach tissues vary widely with the age of the insect. Fat body, midgut, and hindgut tissue showed similar age-dependent changes in levels and activities of these detoxifying enzymes but differences in the three tissues were noted. Standard deviations of data pooled over three to five day periods were negligable and could not be seen graphically so they were not reported.

Fat body tissue showed the greatest enzymatic activity and the greatest variation. Fat body cytochrome P-450 levels (Figure 1) were very low in young adult insects but increased steadily reaching a peak at about 100 days. The levels then fell rapidly approaching those of young insects at about 140 days. Fat body EPN-detoxication (Figure 3) and p-nitroanisole O-demethylation (Figure 4) activities showed curves similar to that of cytochrome P-450 and appeared to parallel the cytochrome content of the tissue. The p-nitrophenol produced was determined by the standard curve shown in Figure 11. NADPH-NT reductase activity (Figure 2) differed in that young insects showed a high capacity to reduce the dye. This activity fell to a minimum at about 60 days but then rose rapidly and, like the other activities, showed a peak at 100 days. Chlorcyclizine injections (60 mg/kg) significantly increased the levels and activities of all fat body enzymes studied. The inductive effect was limited to

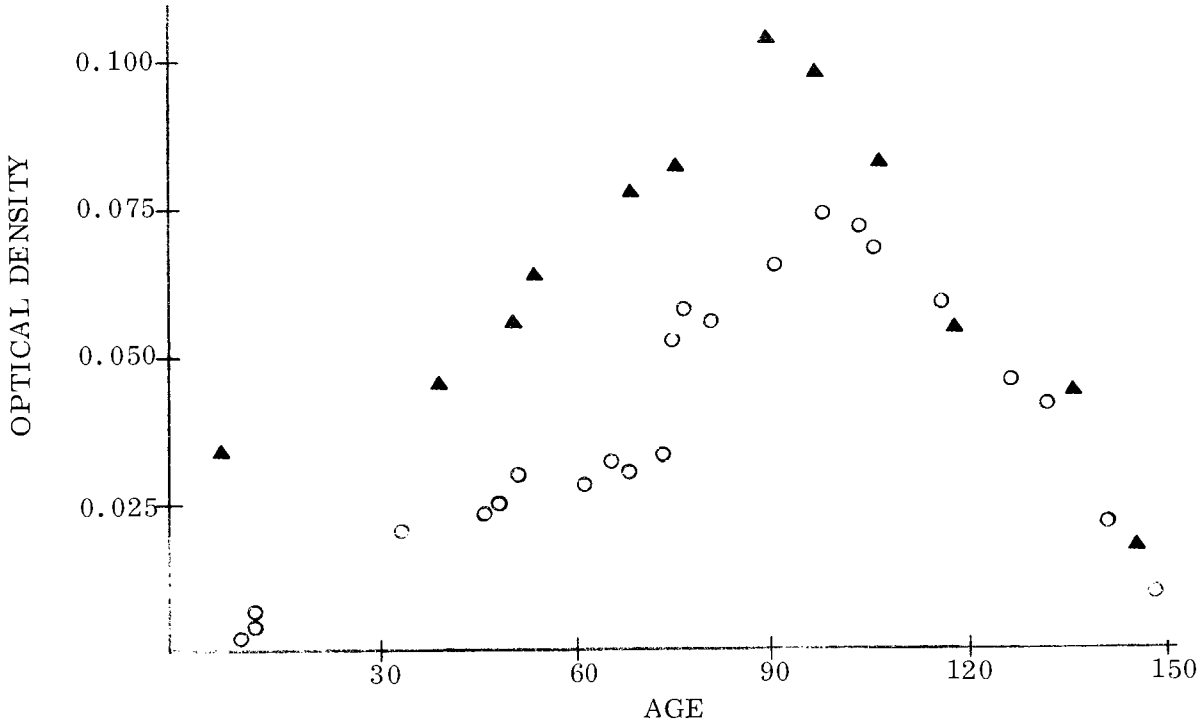


Figure 1. Levels of cytochrome P-450 per mg of protein in (o) normal and (▲) induced female cockroach fat body

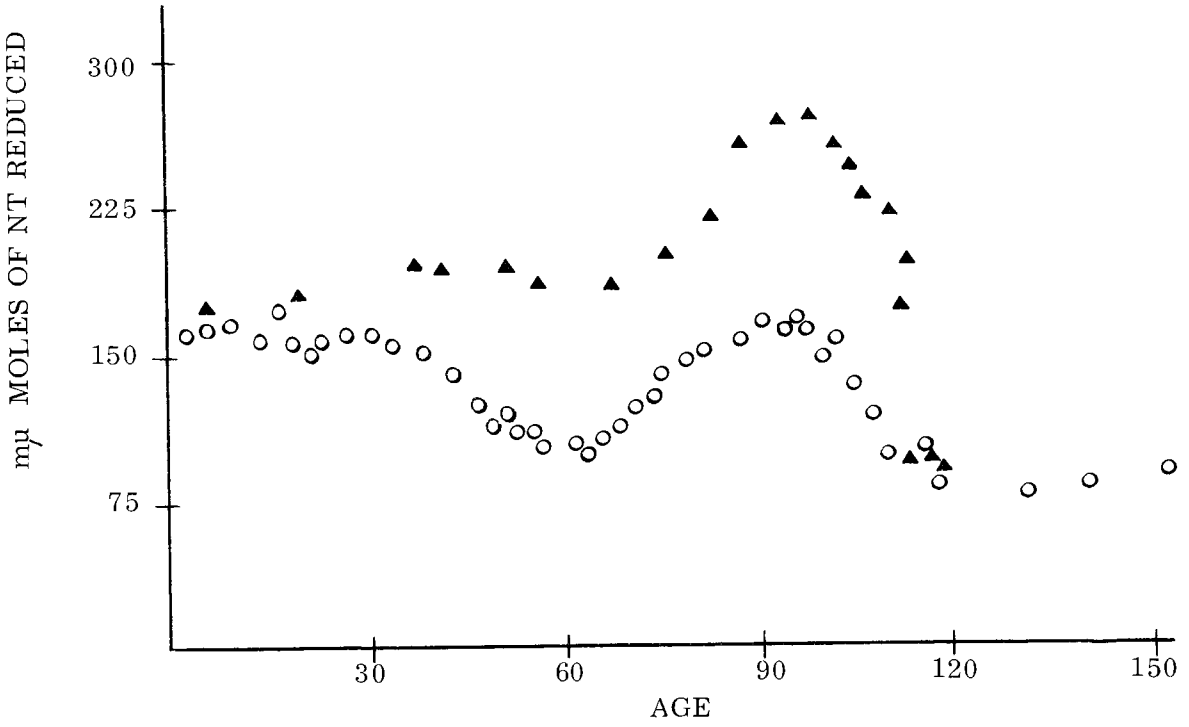


Figure 2. Levels of NADPH-NT-reductase activity per mg protein in (o) normal and (▲) induced female cockroach fat body.

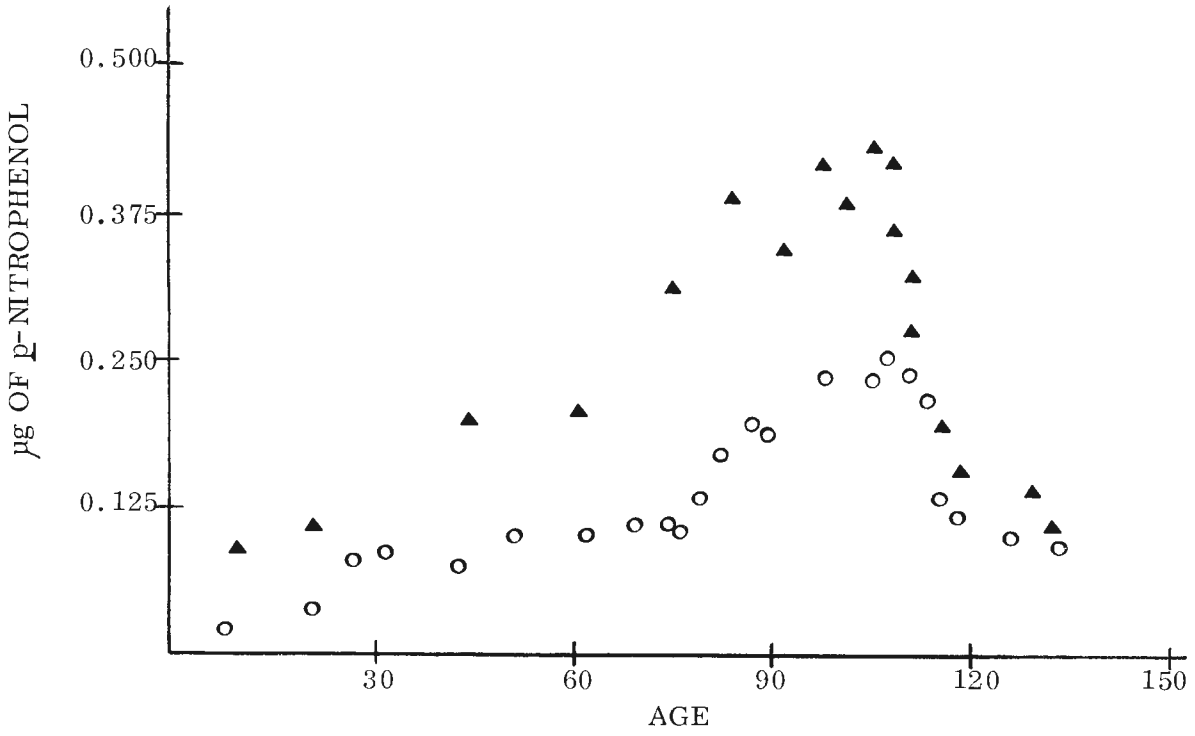


Figure 3. Levels of EPN-detoxication per mg protein in (o) normal and (▲) induced female cockroach fat body.

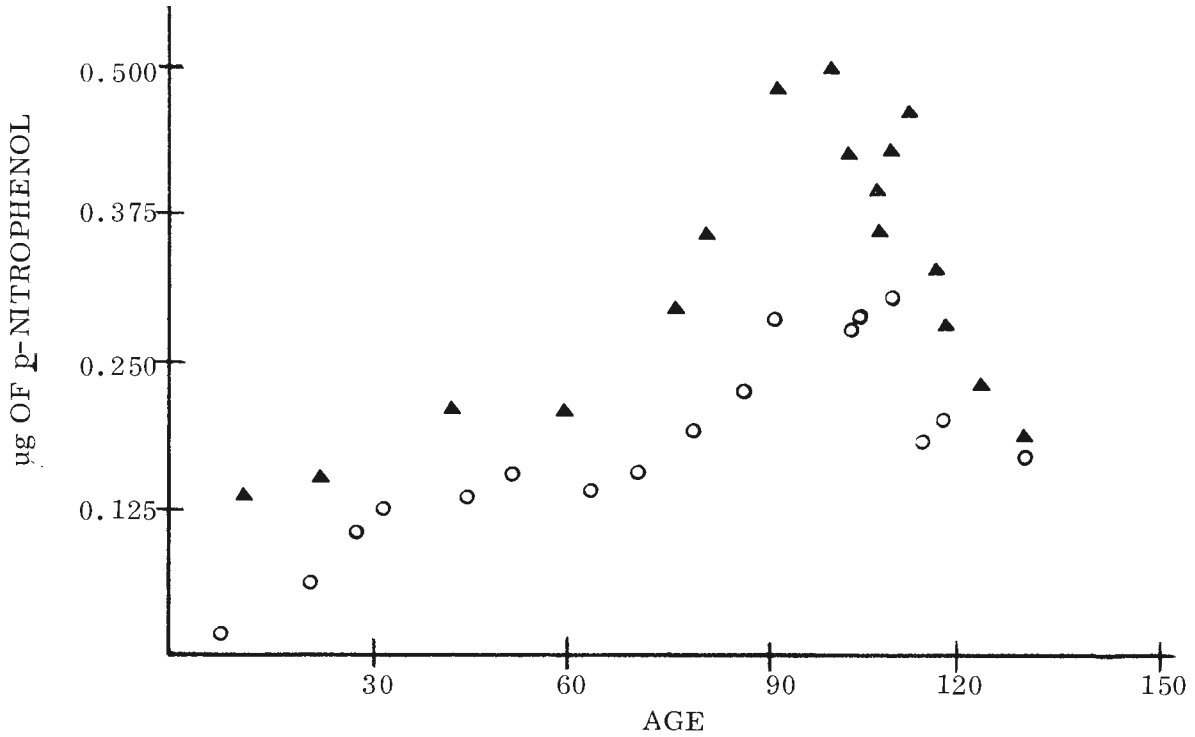


Figure 4. Levels of p-nitroanisole O-demethylation activity per mg of protein in (o) normal and (▲) induced female cockroach fat body.

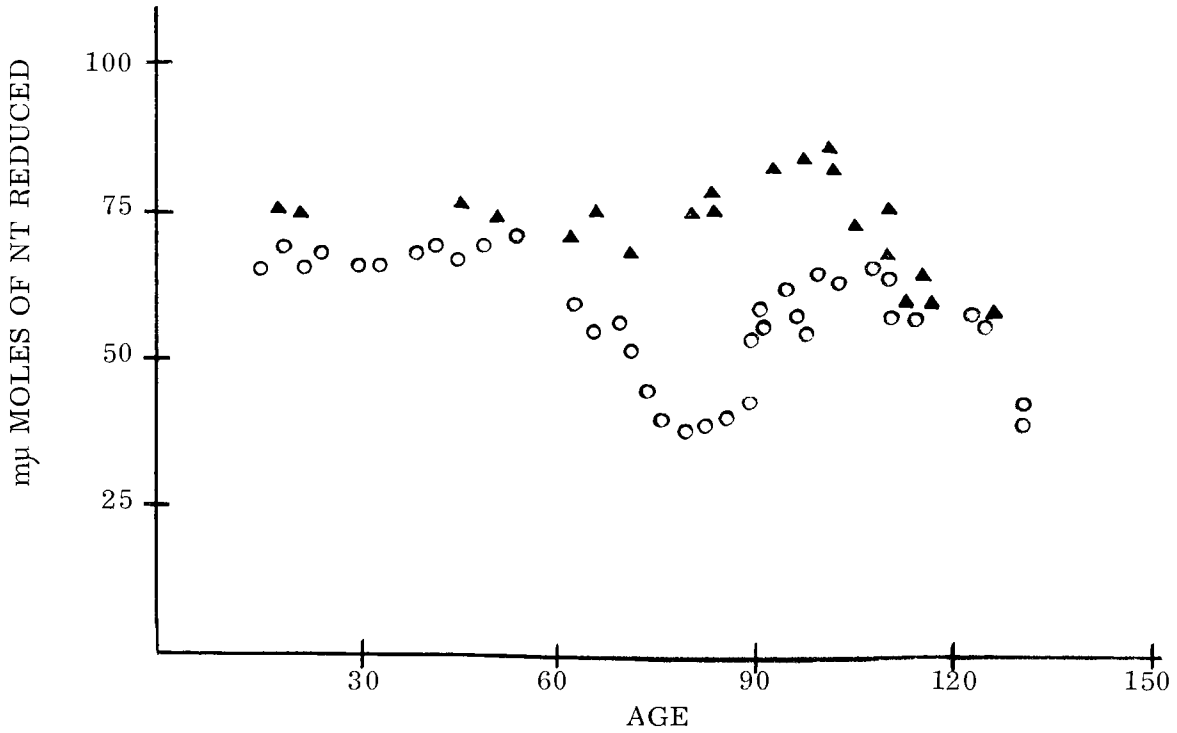


Figure 5. Levels of NADPH-NT-reductase activity per mg protein in (o) normal and (▲) induced female cockroach midgut.

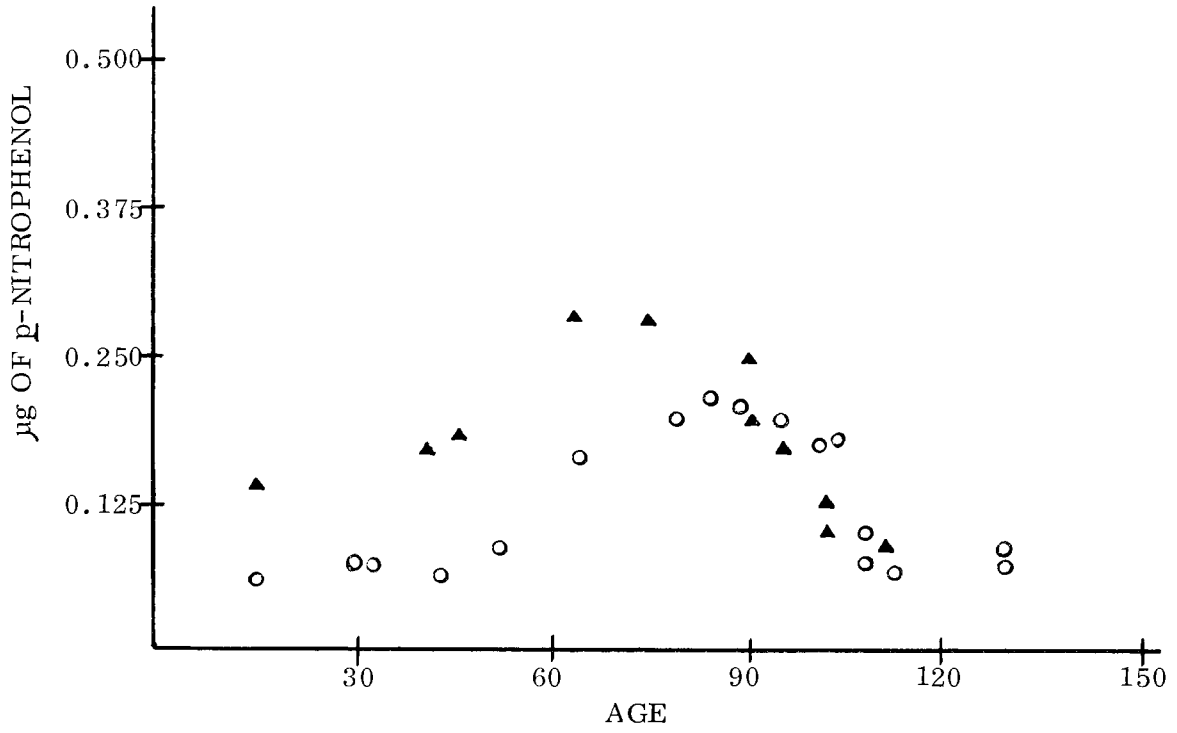


Figure 6. Levels of EPN-detoxication activity per mg protein in (o) normal and (▲) induced female cockroach midgut.

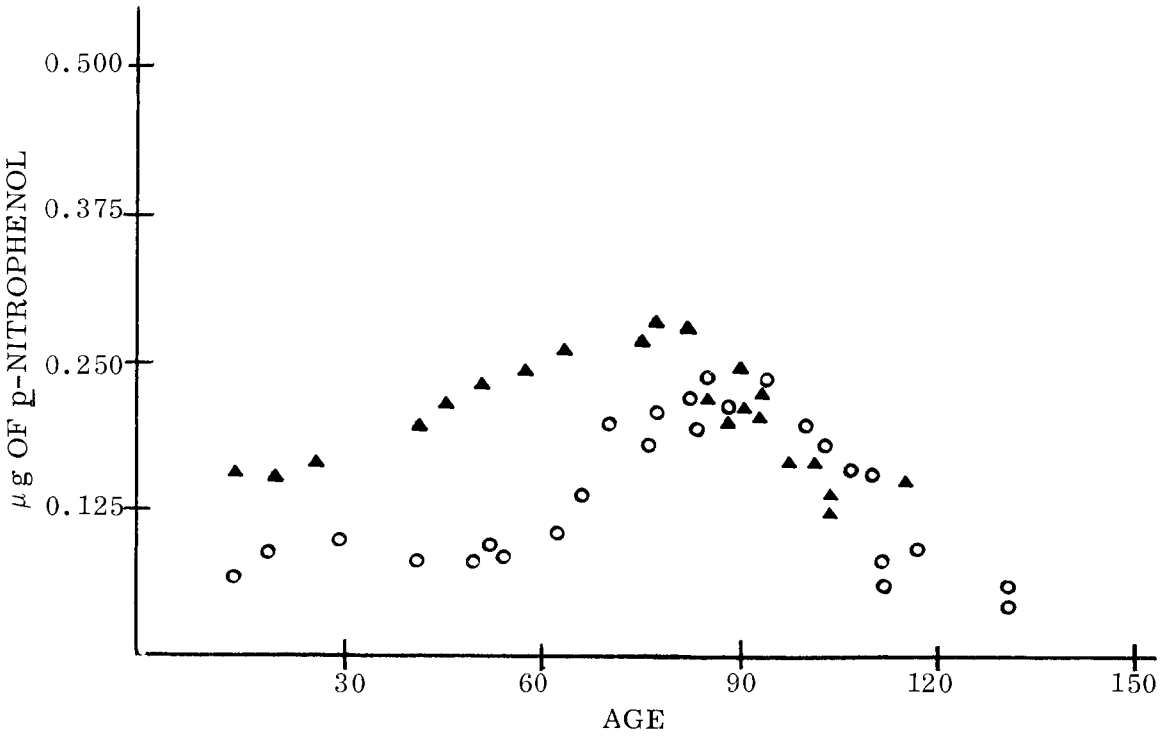


Figure 7. Levels of p-nitroanisole O-demethylation activty per mg protein in (o) normal and (▲) induced adult female cockroach midgut.

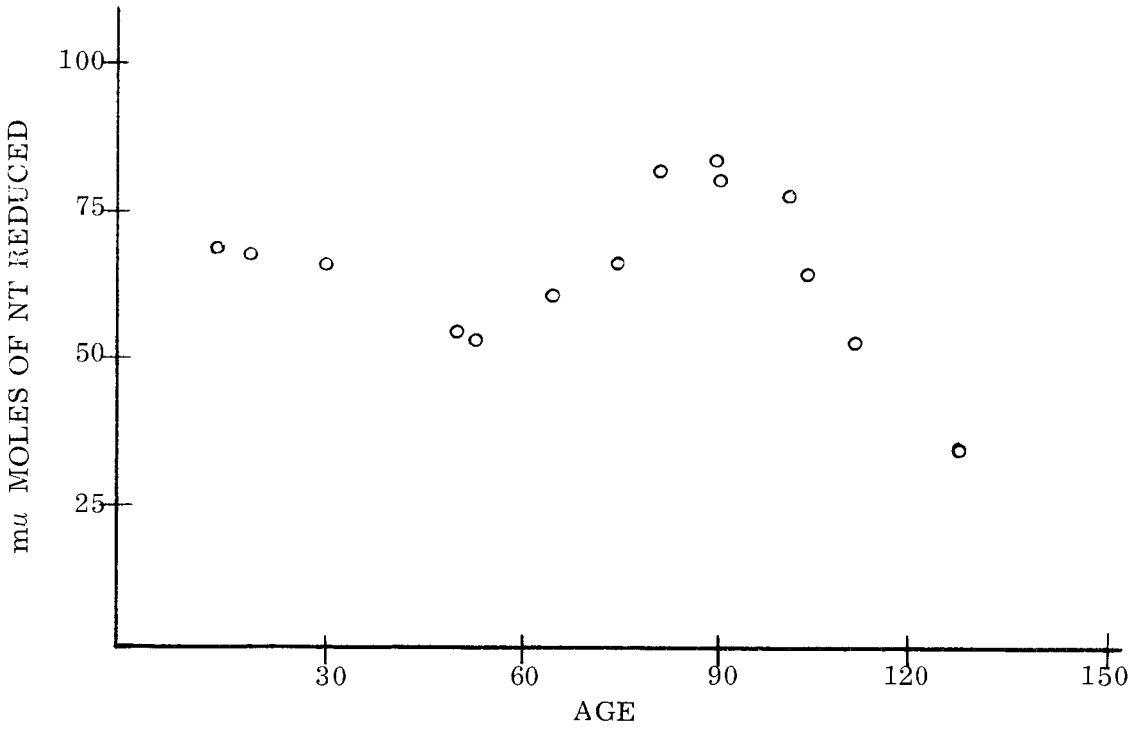


Figure 8. Levels of NADPH-NT-reductase activity per mg protein in normal adult female cockroach hindgut.

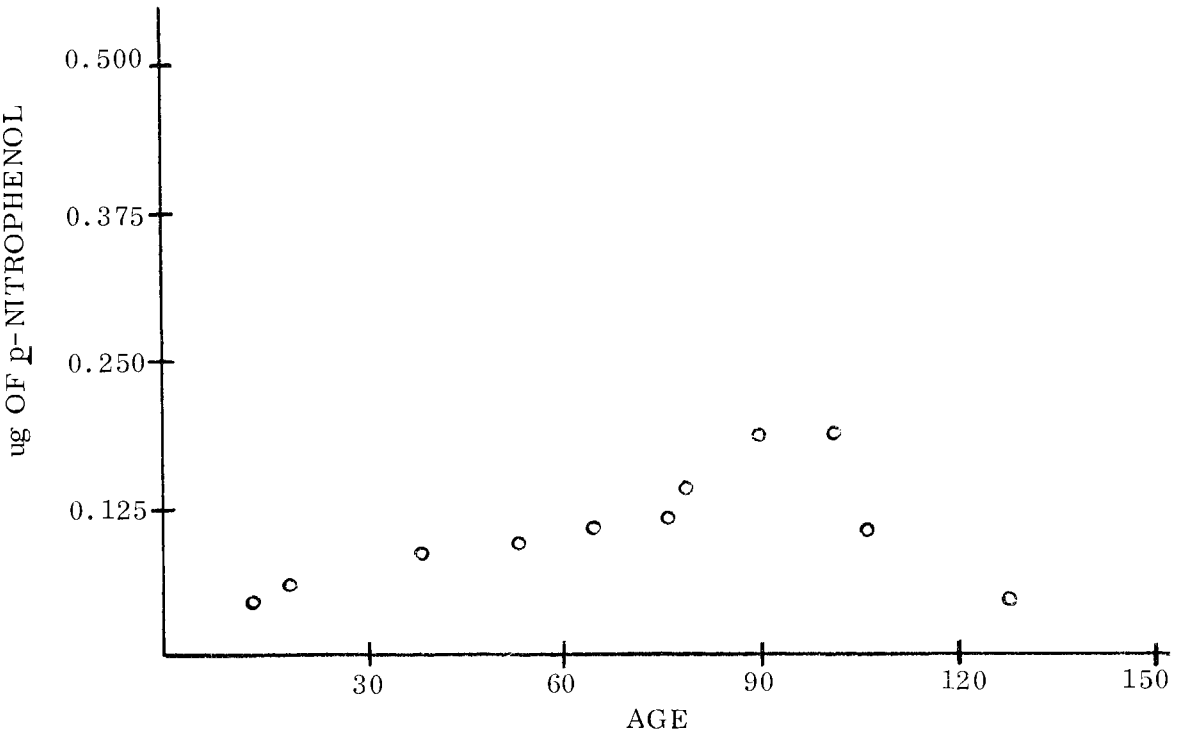


Figure 9. Levels of EPN-detoxication activity per mg protein in normal adult female cockroach hindgut.

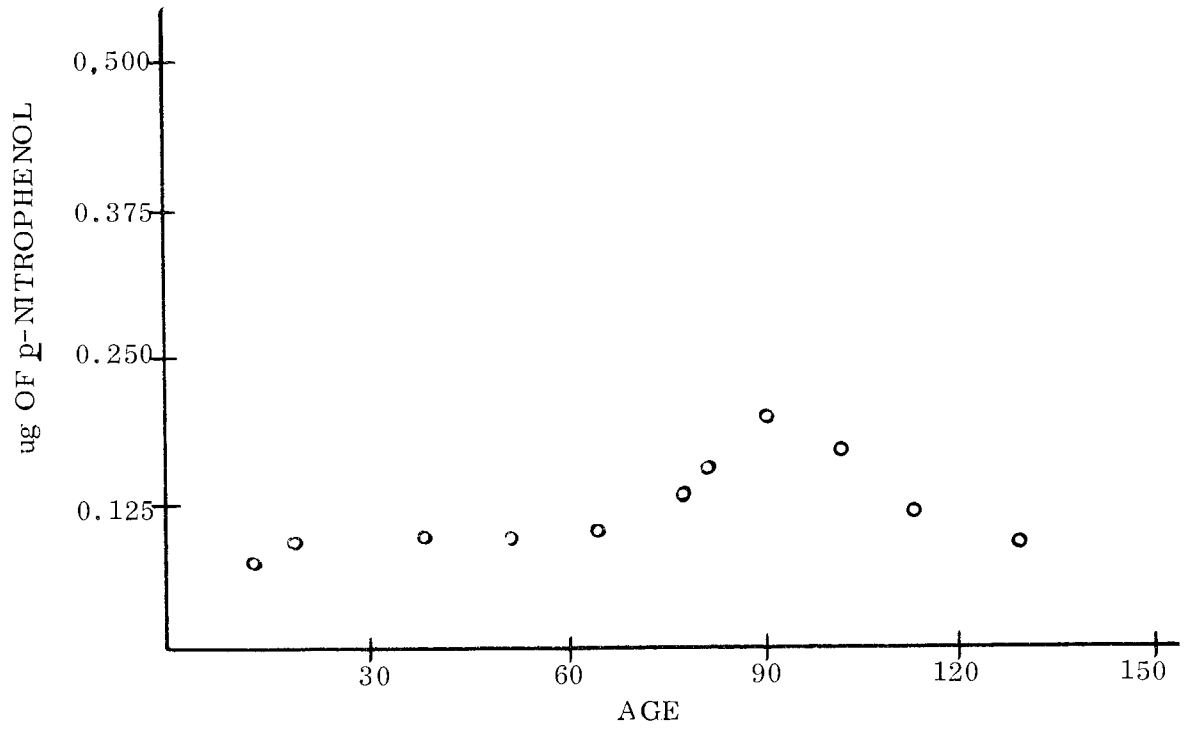


Figure 10. Levels of p-nitroanisole O-demethylation activity per mg protein in normal adult female cockroach hindgut.

insects less than 100 days of age. Beyond this age, when normal (uninduced) enzyme levels were falling, the drug injections had no inductive effect.

Midgut and hindgut tissue showed age-dependent enzyme changes similar to fat body (Figures 5 through 10) but the levels of activity were somewhat lower and the peak of activity occurred at about 90 days or about 10 days earlier than in fat body. Hindgut and midgut showed similar levels of NADPH-NT-reductase activity but hindgut had slightly lower levels of EPN-detoxication and p-nitroanisole O-demethylation activities. As in fat body, chlorcyclizine injections were effective in increasing enzymatic acticity only when normal enzyme levels were rising. Beyond the peak of activity at 90 days normal and induced activities were essentially the same.

Figure 12 shows the standard curve for bovine serum albumin used in making protein determinations. All enzyme activities were standardized relative to one milligram of protein per milliliter of homogenate. Tissue homogenates were prepared so that the final protein concentrations were approximately one milligram per milliliter (10 mg of fat body microsomes per milliliter and two whole midguts or hindguts per 5.75 ml of homogenizing medium).

Figures 13 and 14 show the results of RNA determinations made on normal midgut and hindgut tissues. In both cases total RNA levels were low in young adult cockroaches and reached a maximum at about 90 days of age paralleling the enzyme activities found in these tissues. RNA determinations were made on chlorcyclizine-induced animals as well but no significant differences were found between them and normal insects. RNA levels were

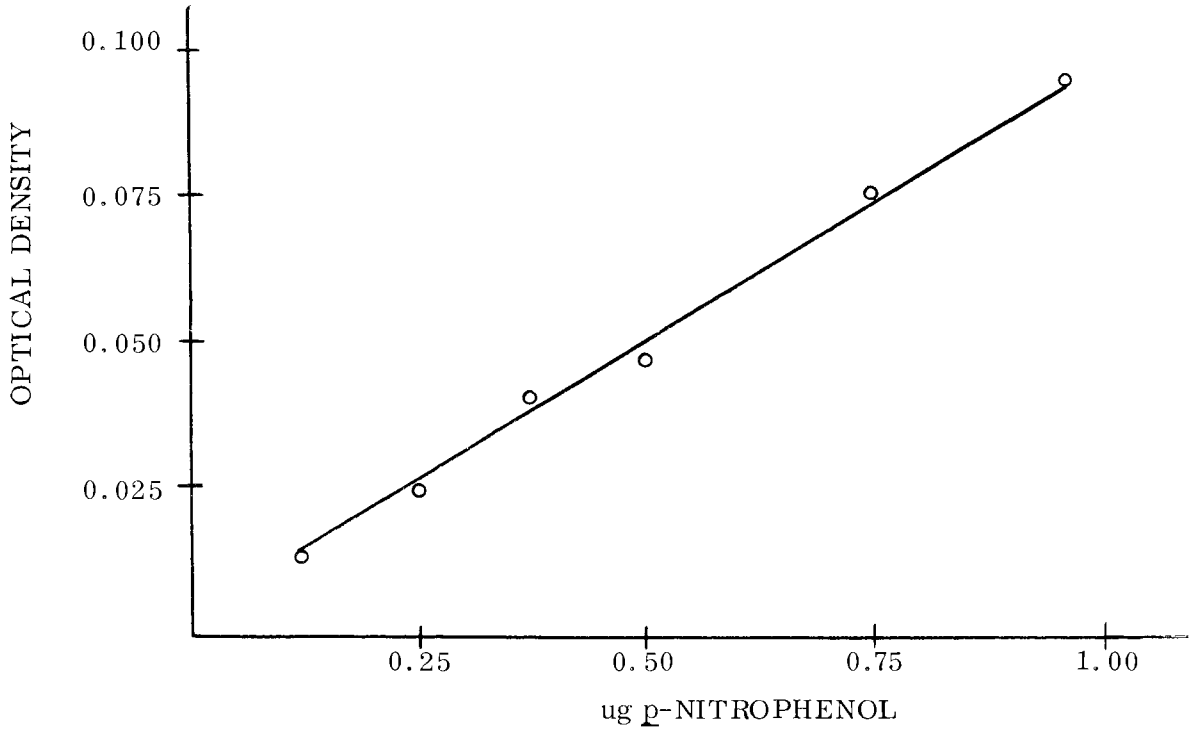


Figure 11. Standard curve for p-nitrophenol.

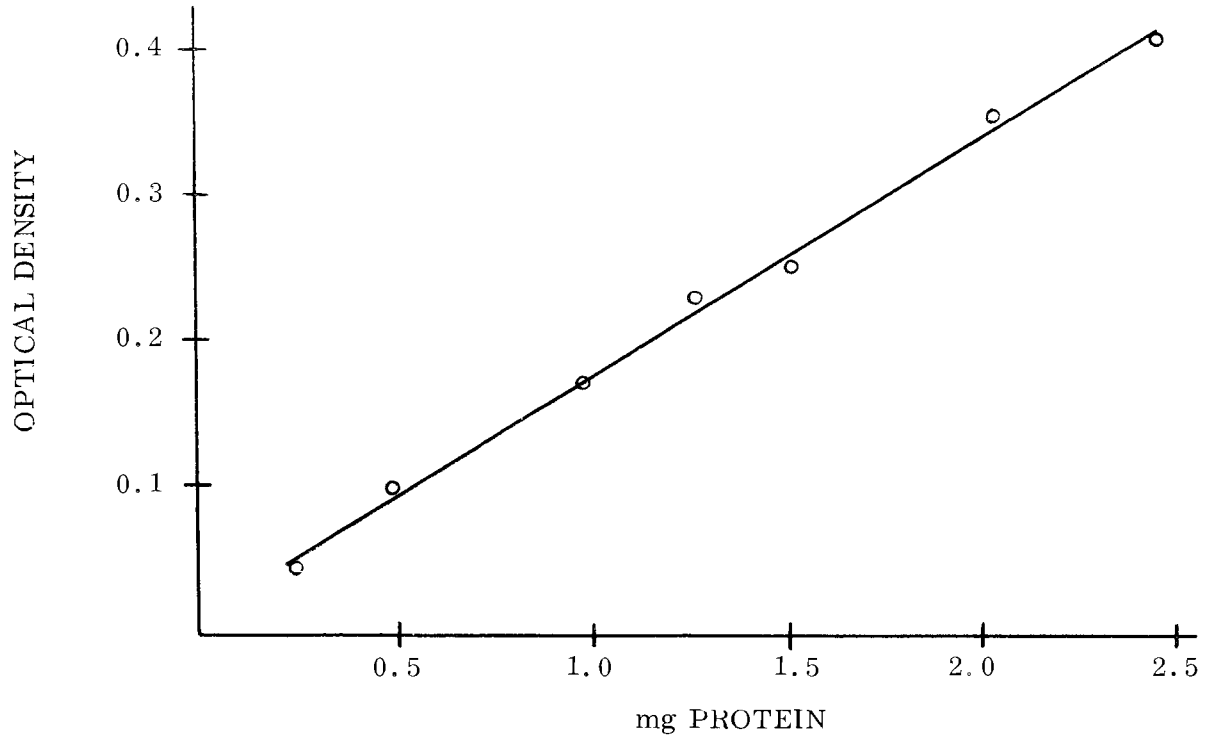


Figure 12. Bovine serum albumin standard curve for protein determinations.

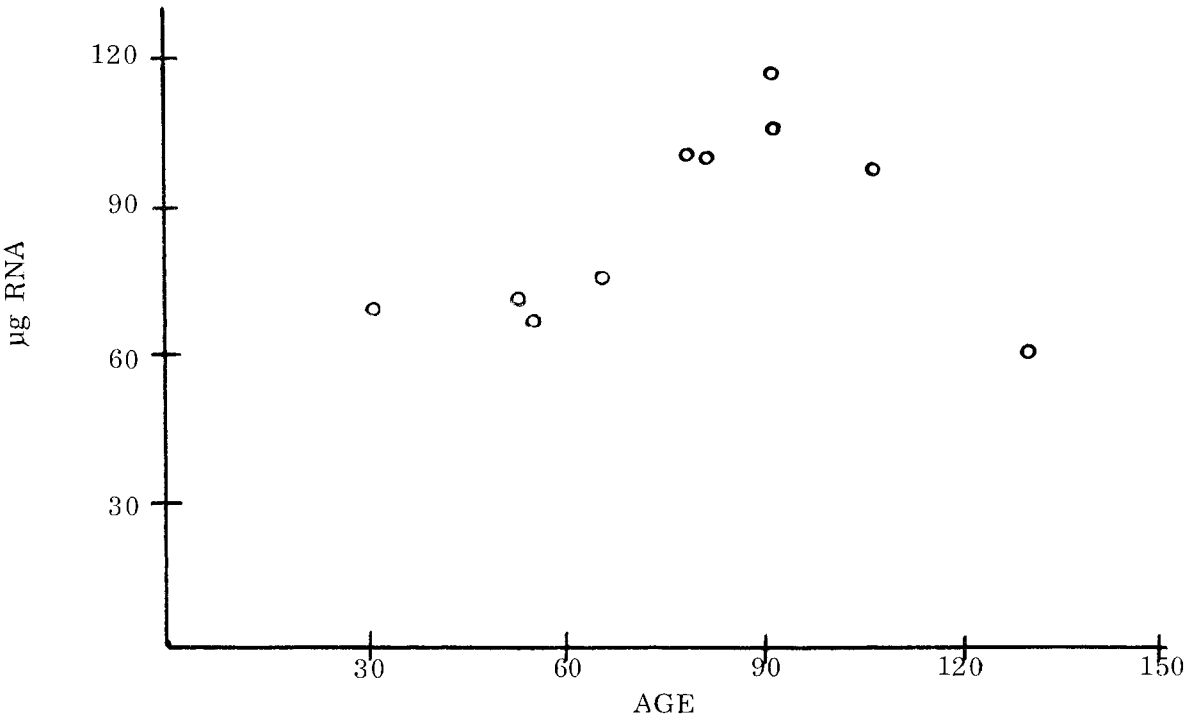


Figure 13. RNA levels per mg protein in normal adult female cockroach midgut.

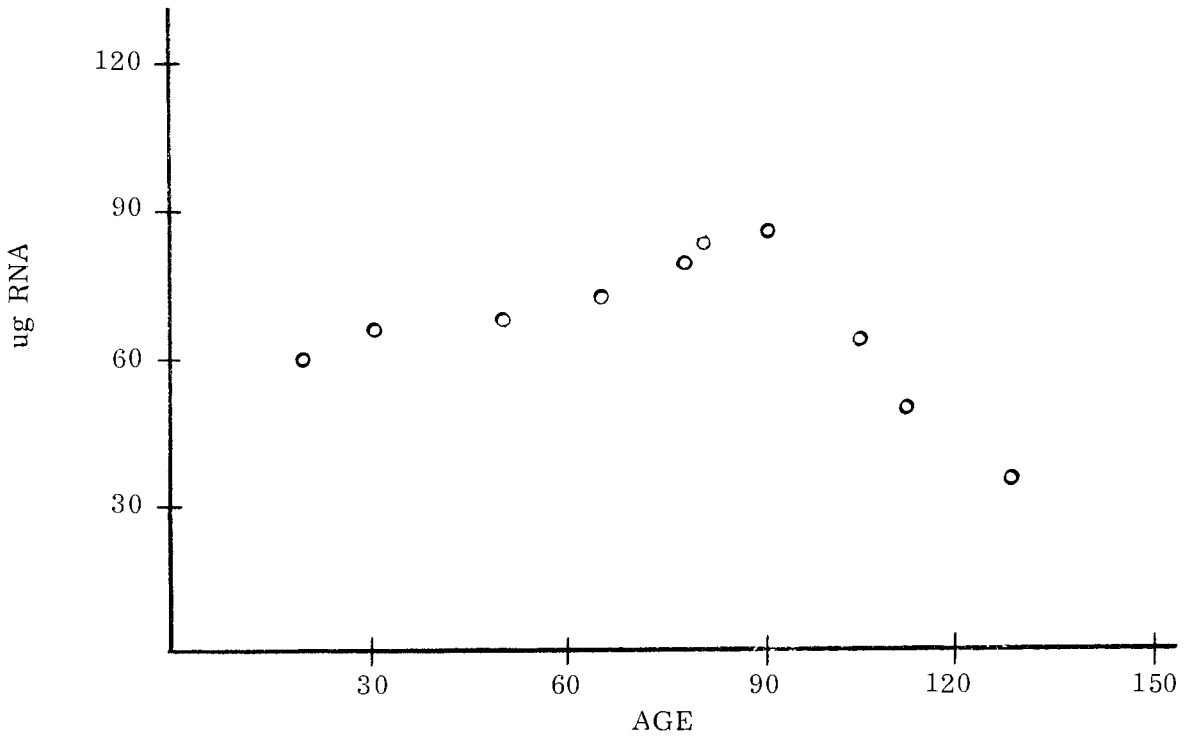


Figure 14. RNA levels per mg protein in normal adult female cockroach hindgut.

standardized using the curve found in Figure 15. No determinations were made in fat body tissue.

Table 1 shows the effects of actinomycin D on chlorcyclizine-induced cockroaches. Surprisingly, administration of the RNA synthesis inhibitor caused increases in the activities of the enzymes studied. Administration of actinomycin D without the inducer gave variable and unpredictable results.

Table 1. Effects of actinomycin D on midgut mixed function oxidase activity

Age	Treatment	NT[a]	EPN[b]	PNA[b]
	none	64	0.072	0.100
27	chlorcyclizine	75	0.138	0.168
	chlor. + act. D	102	0.166	0.228
	none	70	0.085	0.106
52	chlorcyclizine	74	0.182	0.222
	chlor. + act. D	92	0.235	0.238
	none	46	0.205	0.178
69	chlorcyclizine	75	0.266	0.232
	chlor. + act. D	139	0.318	0.292
	none	44	0.230	0.204
85	chlorcyclizine	84	0.266	0.192
	chlor. + act. D	122	0.298	0.280
	none	60	0.175	0.184
101	chlorcyclizine	73	0.166	0.148
	chlor. + act. D	111	0.243	0.226

[a]Activity is expressed as mu moles of NT reduced per mg of protein per 10 minutes incubation.

[b]Activity is expressed as ug of p-nitrophenol produced per mg of protein per 60 minutes.

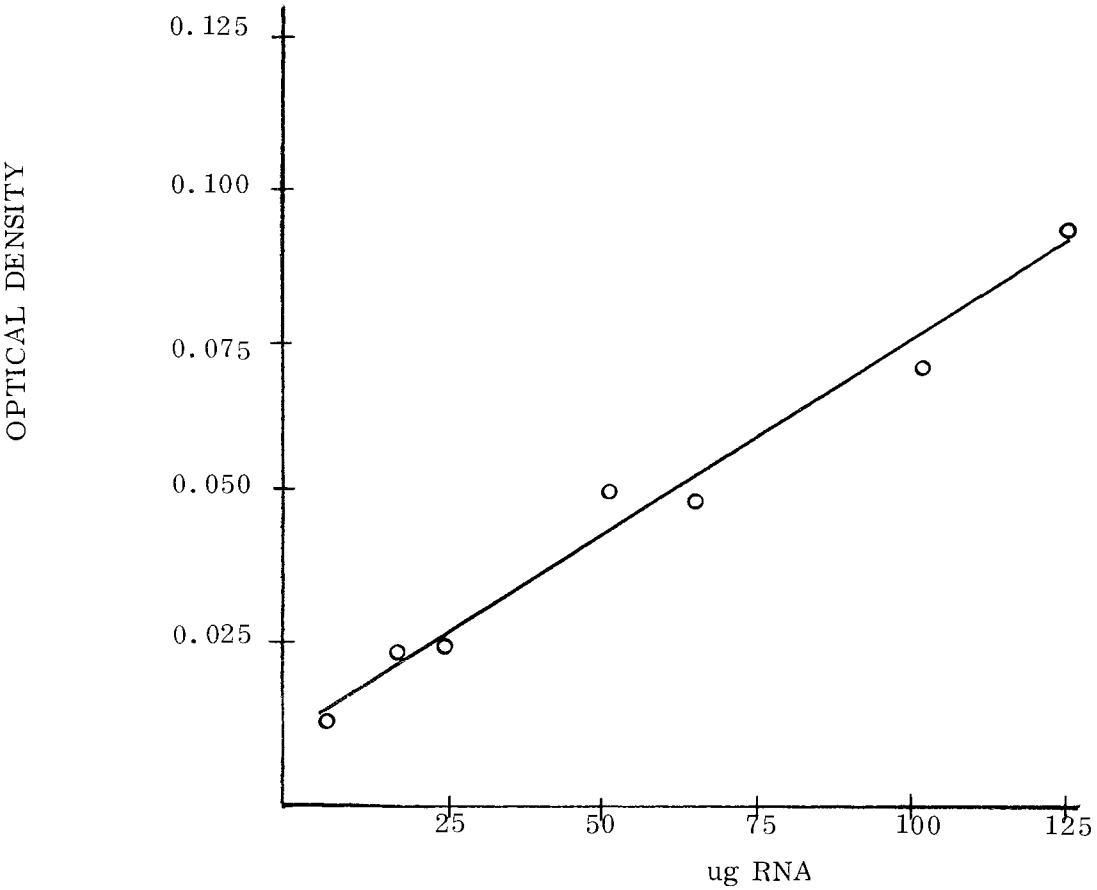


Figure 15. Standard curve for RNA determinations.

Figures 16 through 24 show electron micrographs taken of normal and induced fat body and midgut tissue. Special emphasis was placed on the structure and amount of the endoplasmic reticulum. An effort was made to choose micrographs which were representative of the ranges of structural patterns observed.

Figure 16 shows fat body tissue from a 30 day old untreated female cockroach. Rough endoplasmic reticulum is much in evidence. It is arranged in parallel arrays or lamellae with ribosomes covering the outer surfaces of the membranes. The cisternae are narrow and the complexes appear to be embedded in glycogen-free areas of cytoplasm.

Fat body from 80 day old normal insects (Figure 17) shows endoplasmic reticulum which has become more dispersed. Some semblence of the lamellae remain but many elements appear singly. Ribosomes still cover the outer surfaces of the membranes but some of the cisternae appear to be slightly swollen.

Fat body from normal 100 day cockroaches (Figure 18) shows a sudden and drastic change has occurred in the structure of the endoplasmic reticulum. Although still of the granular form, the membranes have lost all of their lamellar appearance. The cisternae are very swollen and some elements appear tubular in nature. Again the complexes appear embedded in very dense cytoplasm.

Induction of 30 day old insects produces fat body (Figure 19) that is markedly different from normal tissue of the same age. The parallel lamellae

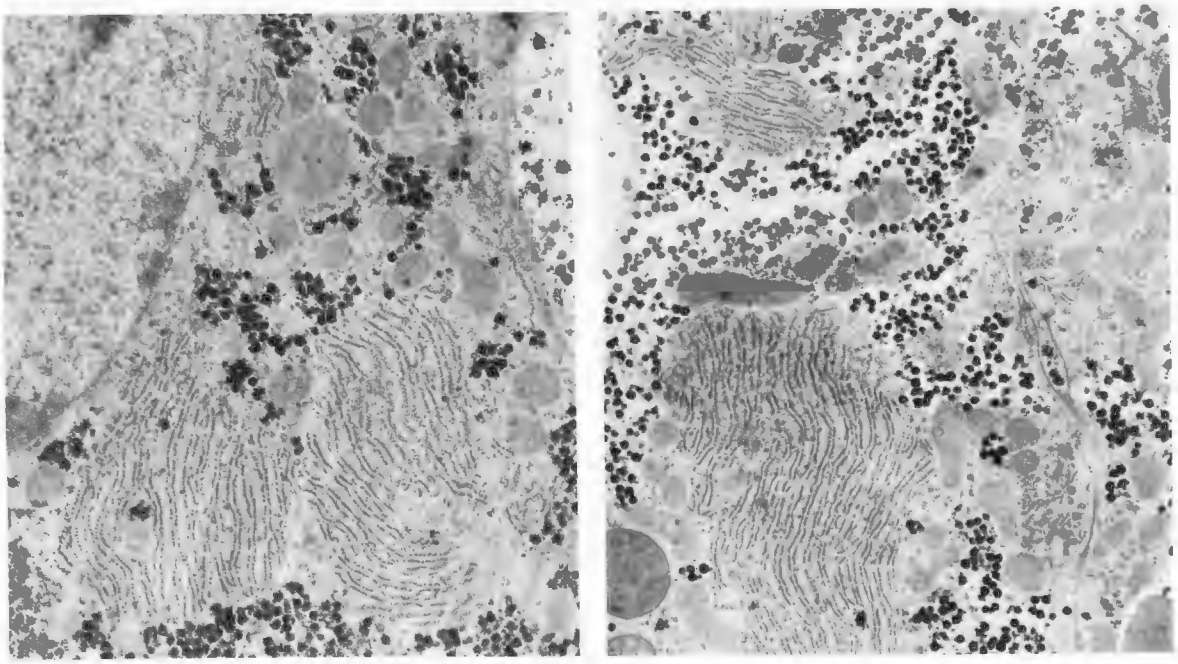


Figure 16. Fat body from a normal 30 day old female (x 11,500).

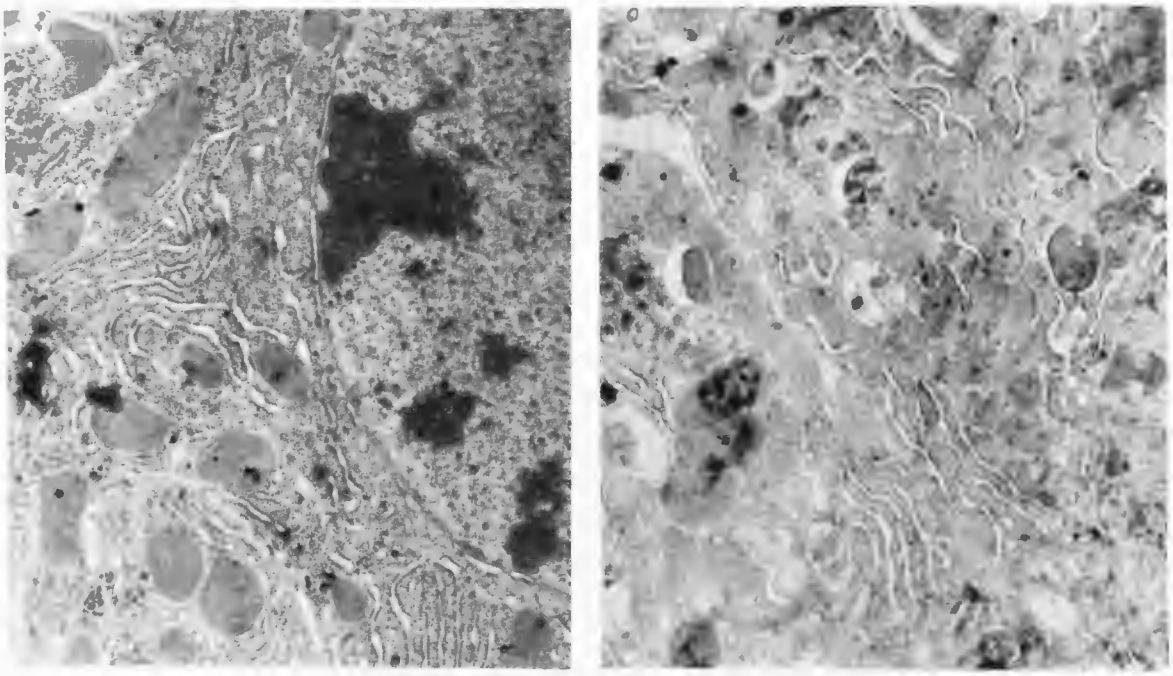


Figure 17. Fat body from a normal 80 day old female (x 12,000).

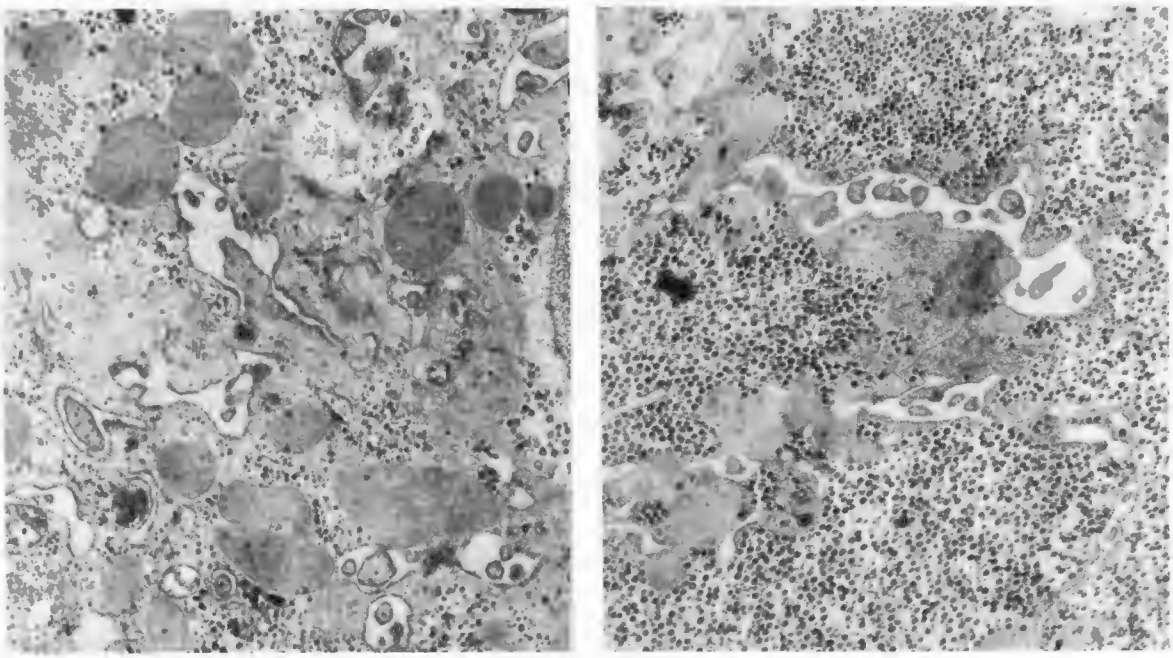


Figure 18. Fat body from a normal 100 day old female (x 10,500).

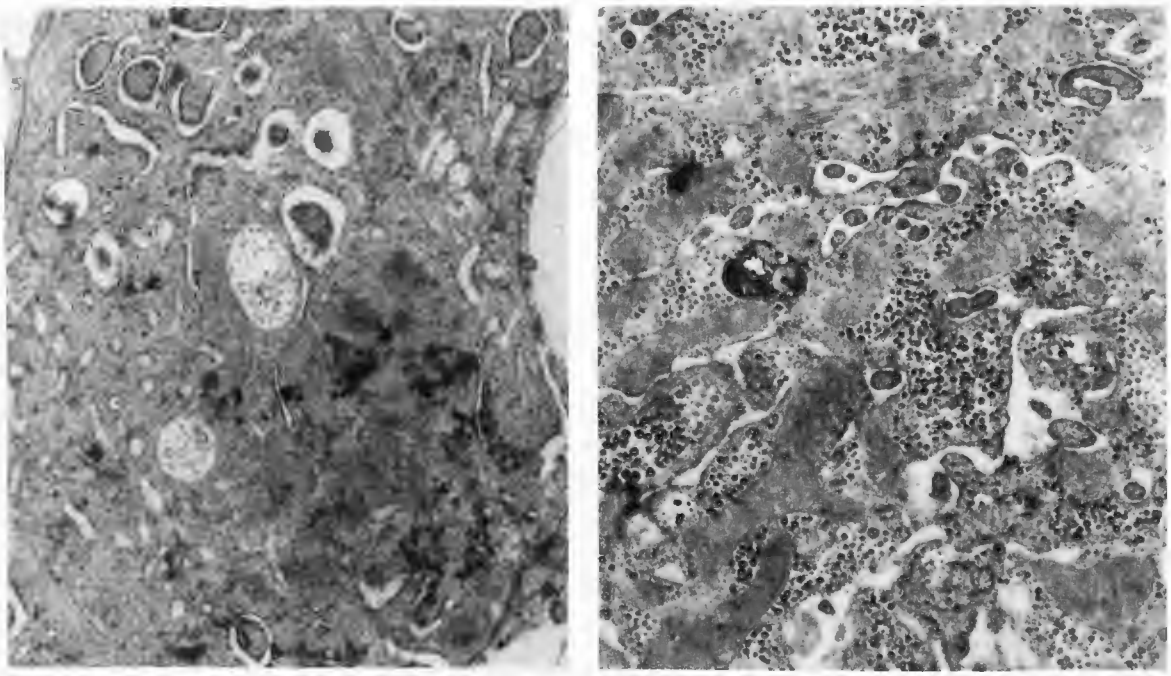


Figure 19. Fat body from an induced 30 day old female (x 12,500).

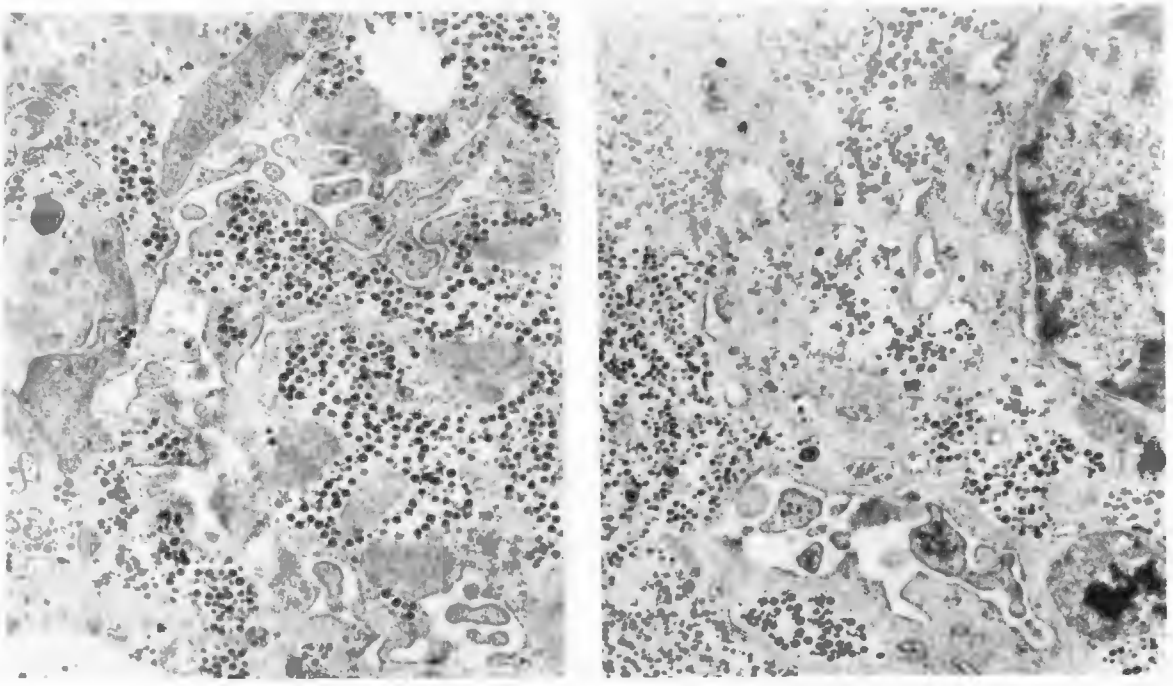


Figure 20. Fat body from an induced 80 day old female (x 11,500).

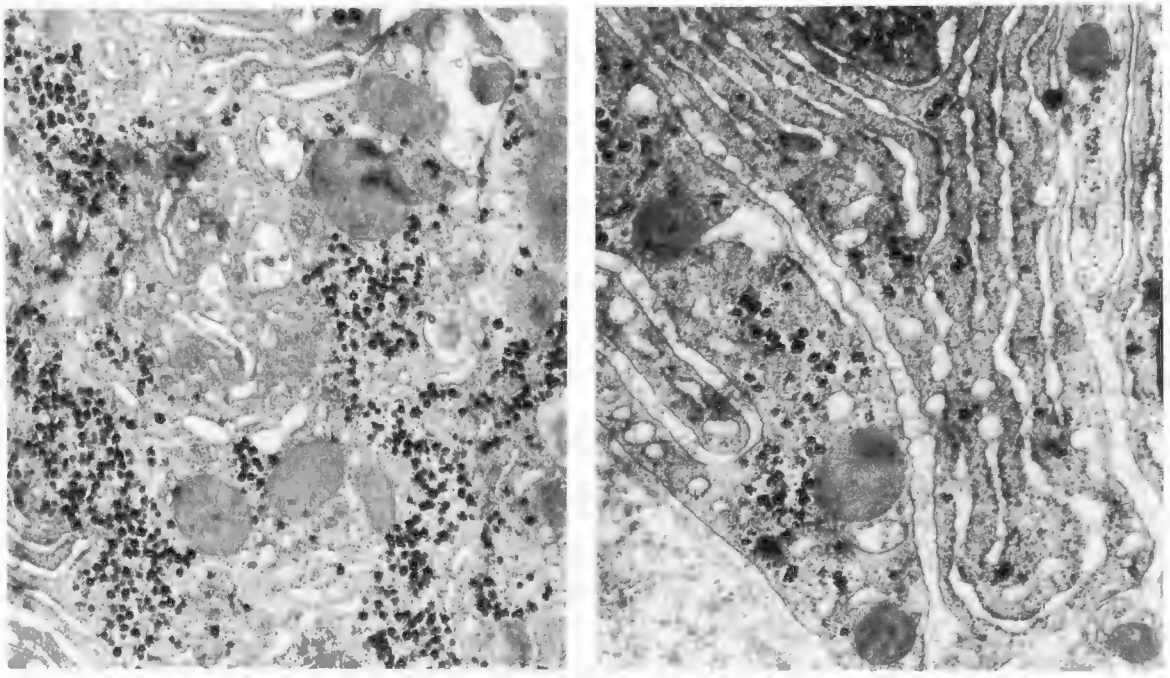


Figure 21. Fat body from an induced 100 day old female (x 16,000).

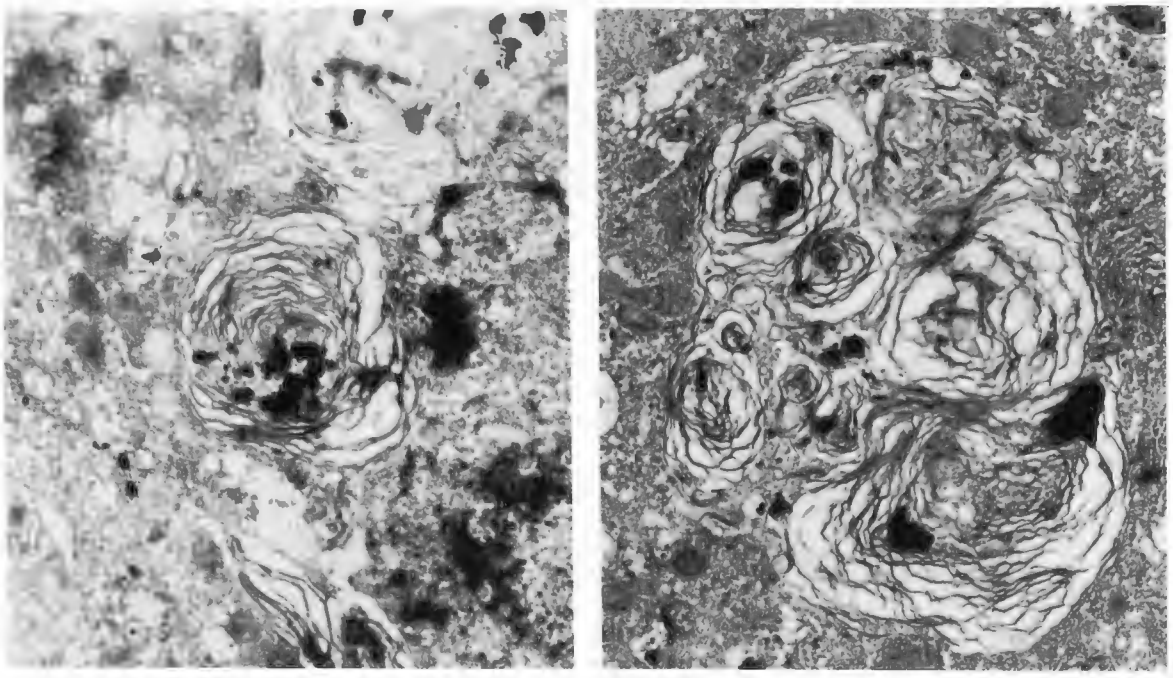


Figure 22. Membrane whorls from fat body of normal 114 day old females showing acid phosphatase activity (x 13,500).

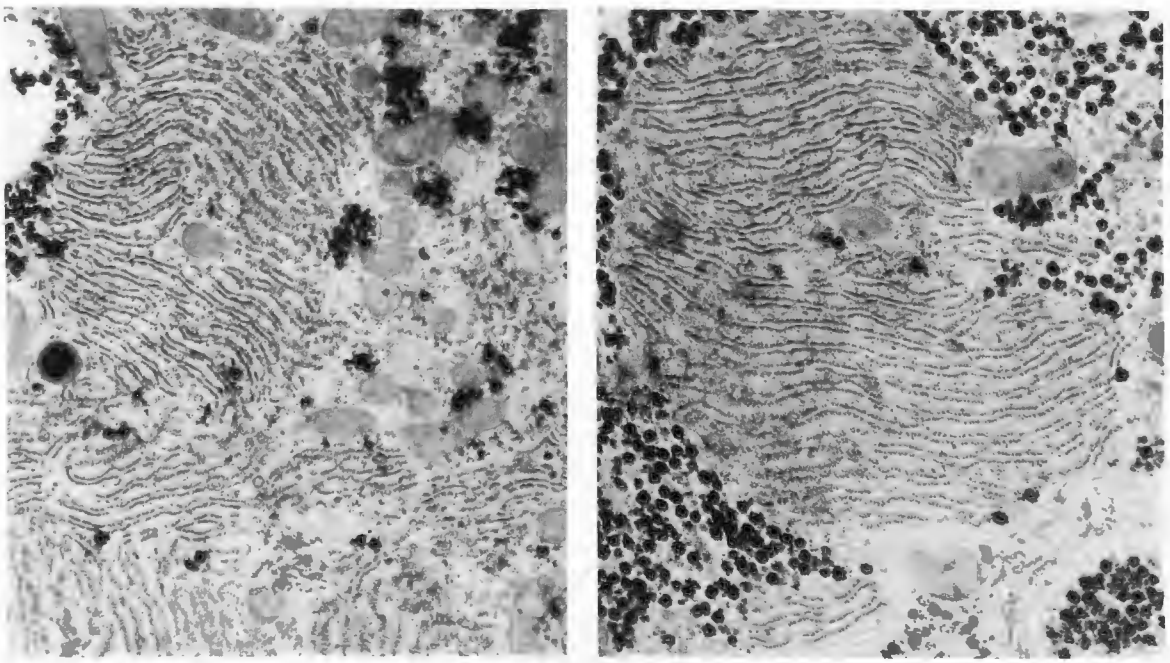


Figure 23. Fat body from a normal 130 day old female (x 11,500).

are not present and some swelling of the cisternae is evident. The endoplasmic reticulum has the appearance of normal 80 or 100 day old tissue.

Fat body from 80 day old induced insects (Figure 20) shows a continuation of the apparent change of the rough endoplasmic reticulum. The tissue appears very similar to that found in normal 100 day old cockroaches. The cisternae are swollen and irregular. Even the cisternae of the nuclear envelope appear to be somewhat enlarged.

Figure 21 shows fat body from induced insects 100 days old which had the highest enzymatic activity of any tissue examined. The endoplasmic reticulum is becoming tubular in form. Many of the tubular elements are losing or have lost their ribosomes and have the appearance of typical smooth reticulum. Even some of the lamellar formations appear to be devoid of ribosomes.

Figure 22 shows fat body from normal 114 day old insects which has been selectively stained to show acid phosphatase activity. Although the pictures are of rather poor quality due to the staining procedure, very little endoplasmic reticulum was found. Instead, the cytoplasm was filled with large membrane whorls which showed a positive acid phosphatase activity. Some whorls were quite small having only a few membrane layers while others, as those pictured, were very large and complex. The whorls were not found in any particular area of the cell but seemed to be scattered randomly.

Figure 23 shows normal fat body from 130 day old cockroaches. Enzyme activities in these insects are approaching those of young adults and the endoplasmic reticulum has the appearance of that found in 30 day old cockroaches (Figure 13). The membranes again are entirely rough and arranged in lamellar

formation. While not pictured, membrane whorls were present and appeared to be larger and more complex than those found in 114 day old insects.

Similar age-dependent development of the endoplasmic reticulum was found in midgut tissue. Figure 24, of midgut from normal 30 day old insects, shows endoplasmic reticulum which is rough, lamellar, and very abundant much like that found in fat body of the same insects.

Figure 25 shows midgut from a normal 90 day old adult. Rough endoplasmic reticulum is still abundant but many smooth profiles are present. Smooth and rough membranes appear to be present in about equal amounts. The rough reticulum still has some lamellar arrangement but single elements can be seen.

Figure 26, showing midgut from normal 105 day old cockroaches, indicates a quite dramatic change has occurred. Much of the endoplasmic reticulum has disappeared. Smooth vesicles are gone entirely. A few rough strands are present but they are widely dispersed and show no lamellar appearance. Membrane whorls are again present and are very compact. Figure 27, of midgut 10 days older, indicates that as in fat body tissue the whorls have acid phosphatase activity. The whorls appear more diffuse than those in 105 day old tissue but this may be due to the staining procedure. The phosphatase activity appears to be concentrated in cytoplasmic inclusions within the whorls rather than on the membranes themselves.

Midgut from induced 30 day old insects (Figure 28) shows characteristics very similar to those found in normal 90 day old animals. Both rough and smooth reticulum are present. The cisternae of the rough strands appear

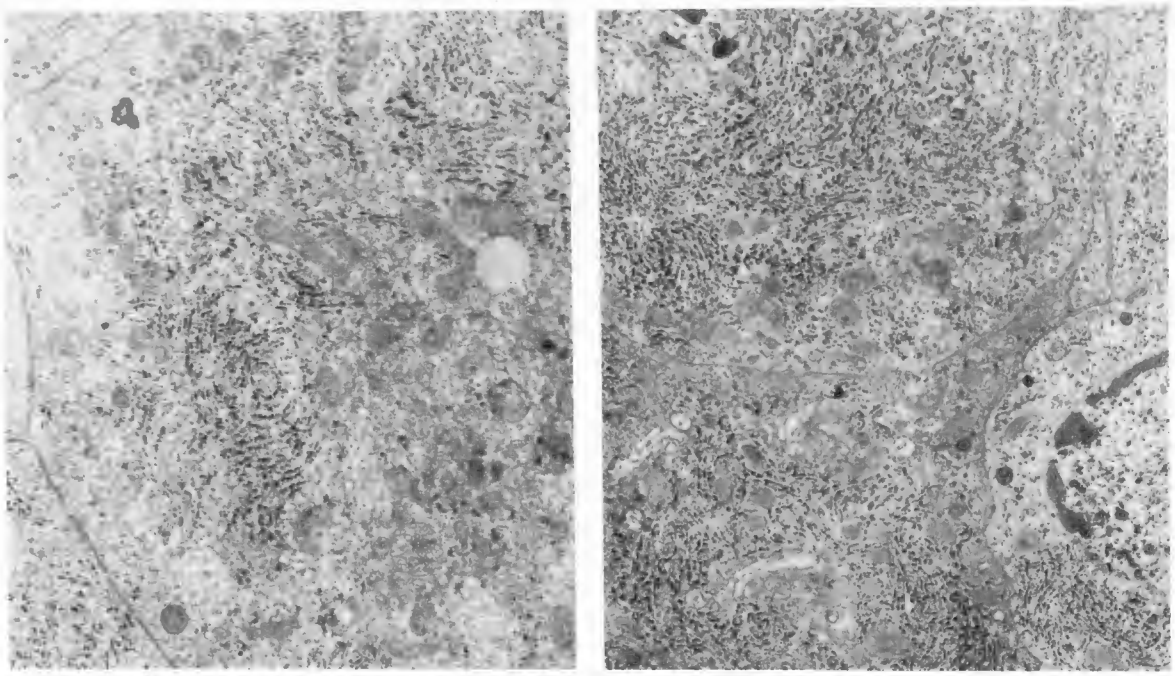


Figure 24. Midgut from a normal 30 day old female (x 13,500).

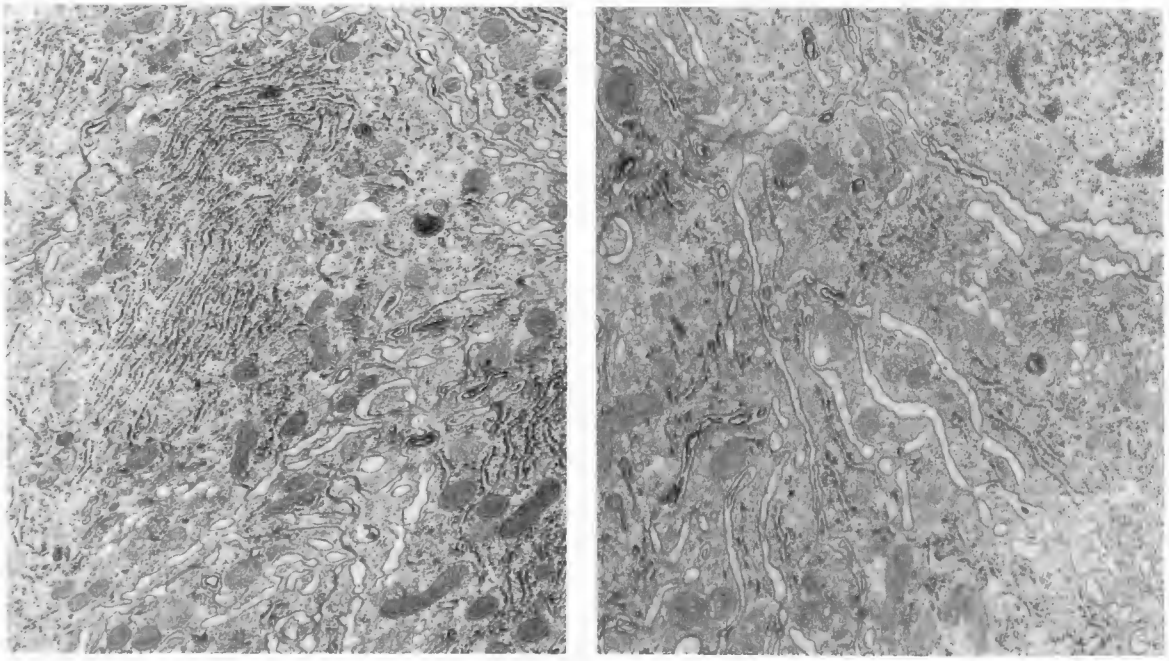


Figure 25. Midgut from a normal 90 day old female (x 13,500).

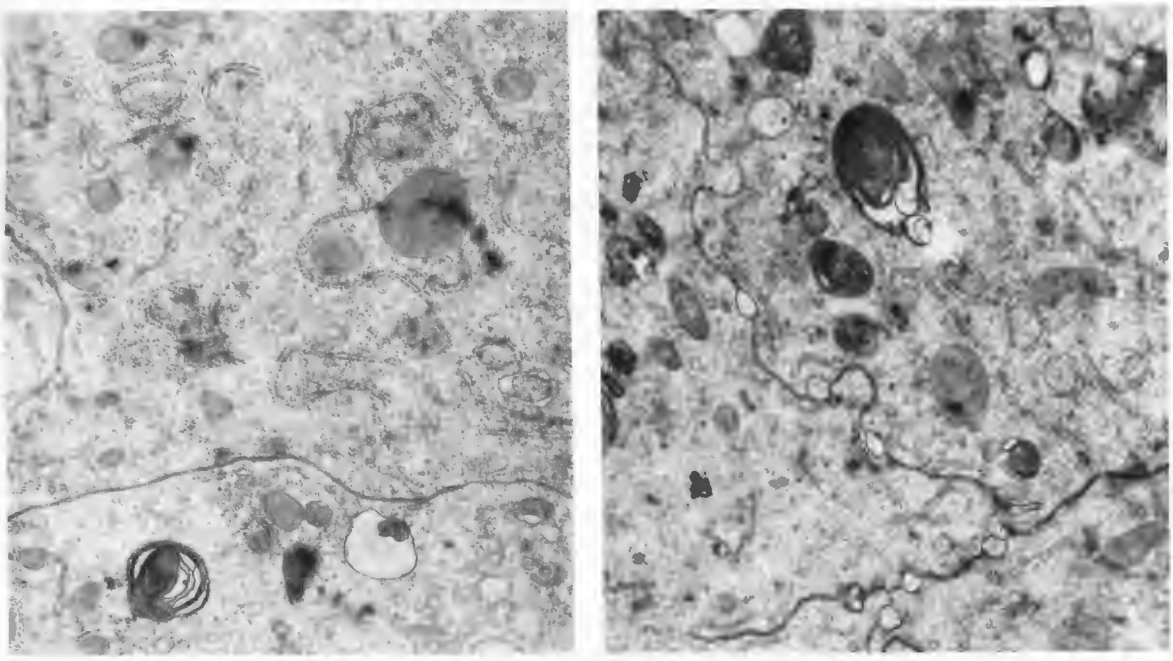


Figure 26. Midgut from a normal 105 day old female (x 15,000).

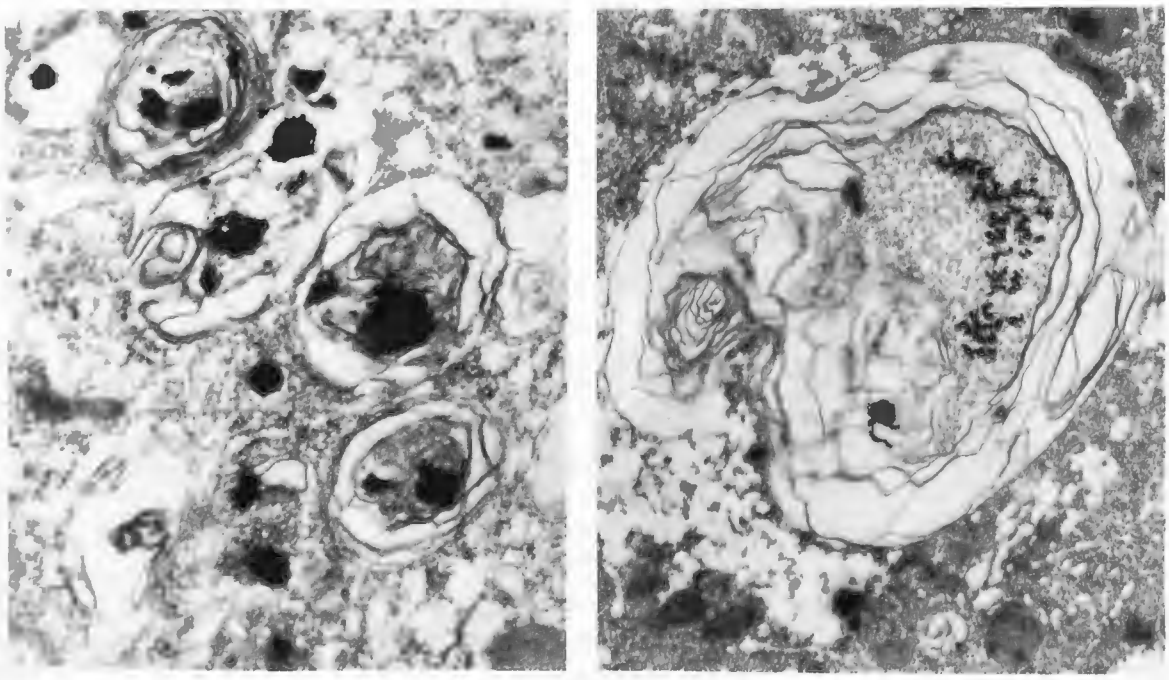


Figure 27. Midgut from normal 115 day old females with membrane whorls showing acid phosphatase activity (x 35,000 and 17,500).

slightly swollen and have little or no parallel arrangement. Figure 29 of midgut from 80 day induced insects is similar except that more smooth and less rough endoplasmic reticulum appears to be present. A few small membrane whorls also appear to be forming. There was also some evidence of free ribosomes in the cytoplasm.

Induction of 90 day old insects produces tissue (Figure 30) that is nearly devoid of rough endoplasmic reticulum. Smooth profiles are very abundant as are many small membrane whorls. At higher magnification the whorls appear to be located within the cisternae of the smooth reticulum. Figure 32 shows the same tissue stained for acid phosphatase activity. It appears that the activity is also located within the cisternae of the smooth reticulum although none of the small whorls are apparent.

Figure 31 shows midgut tissue from 105 day old induced insects. The smooth reticulum is gone, a few strands of the rough are present, and tightly packed membrane whorls are commonly seen. The tissue is almost exactly like that of normal tissue of the same age.

Figure 33 from induced 116 day old insects again shows the structure of a large membrane whorl stained for acid phsophatase activity. The whorl is complex and appears to contain cytoplasmic inclusions which show the phosphatase activity.

Figure 34 shows that midgut from uninduced 130 day old insects is similar to that found in young insects (Figure 24) except for the presence of the membrane whorls which may be very large, tightly wound and complex. Rough

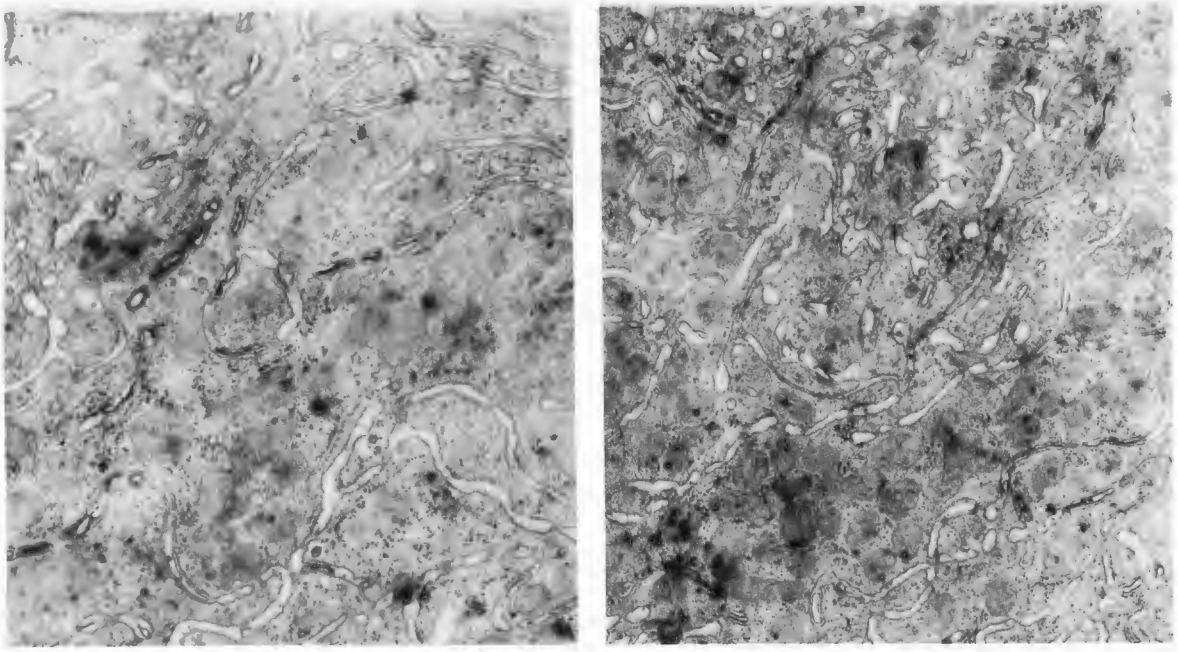


Figure 28. Midgut from an induced 30 day old female (x 15,000).

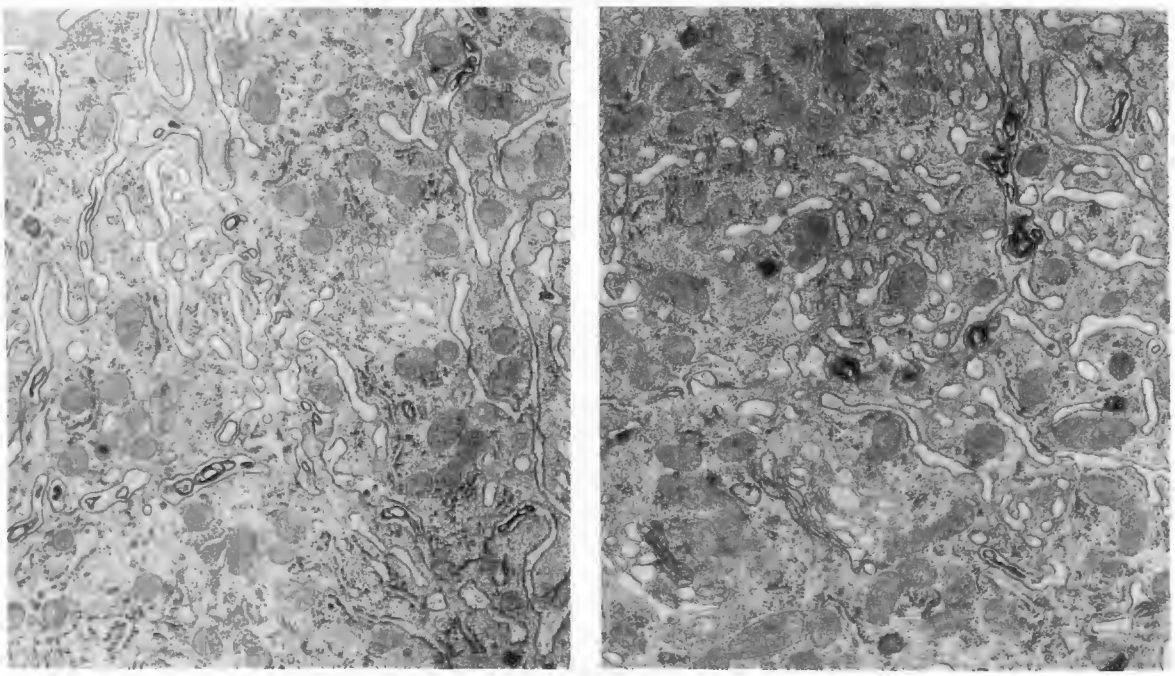


Figure 29. Midgut from an induced 80 day old female (x 15,000).

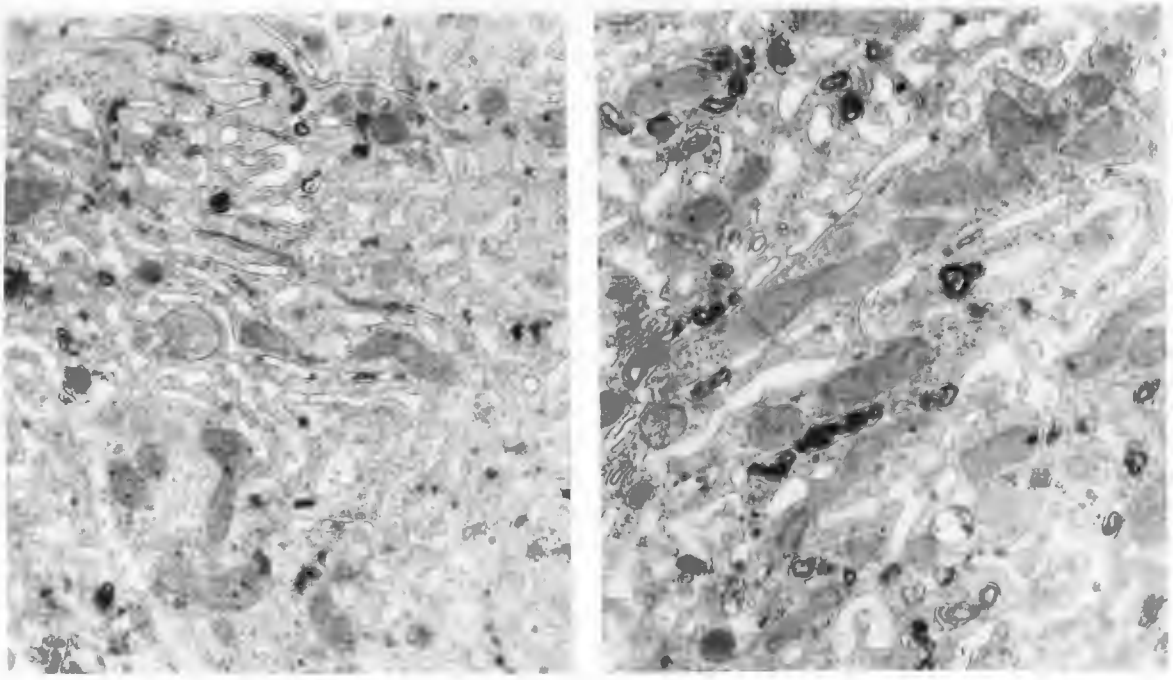


Figure 30. Midgut from induced 90 day old females (x 13,000 and 29,000).

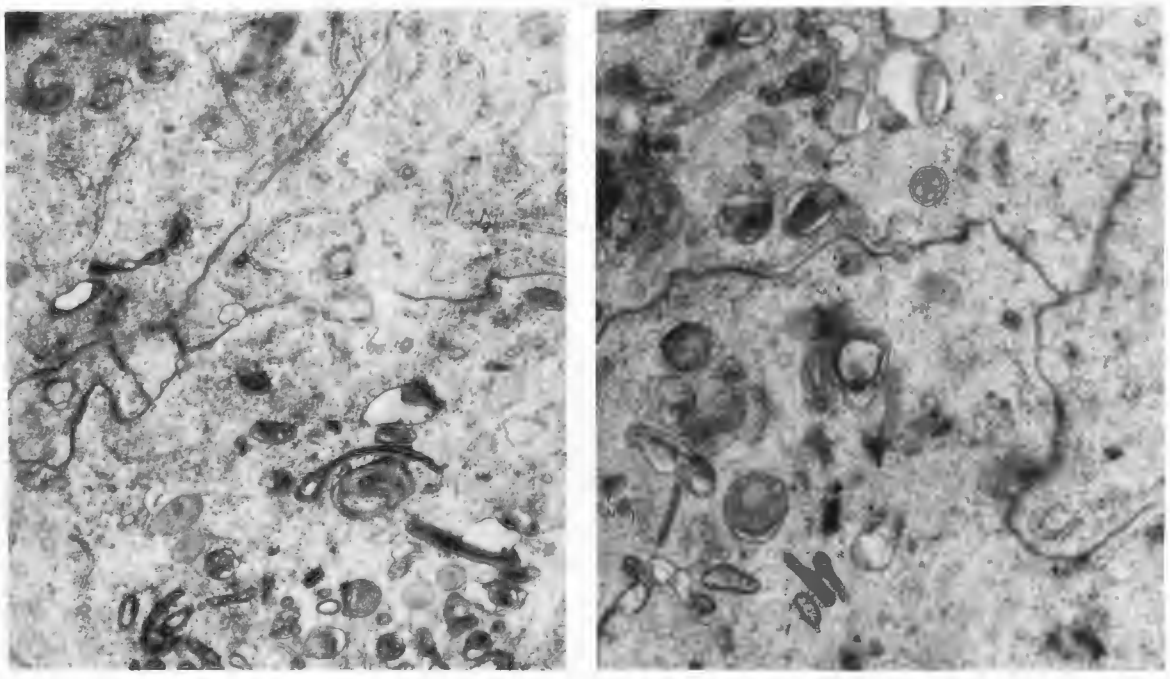


Figure 31. Midgut from induced 105 day old females (x 15,000).

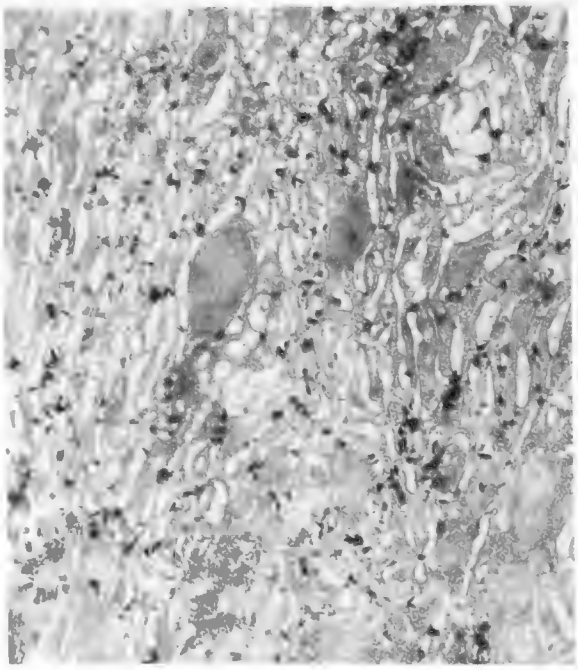


Figure 32. Induced 90 day midgut (x 14,500).

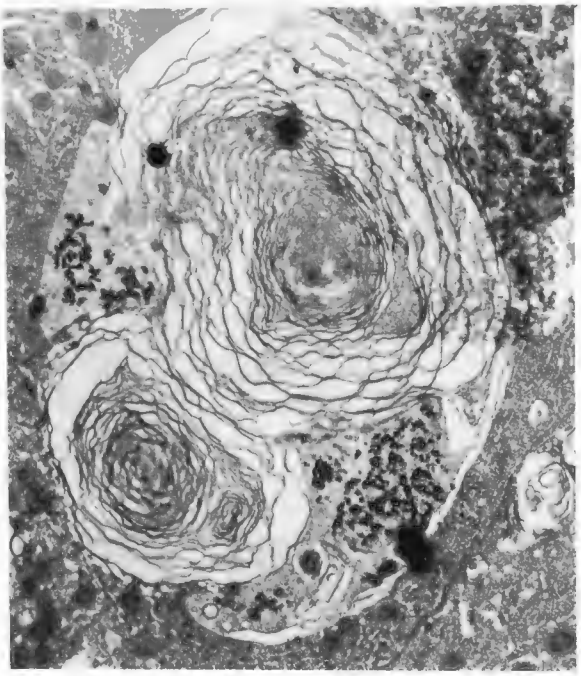
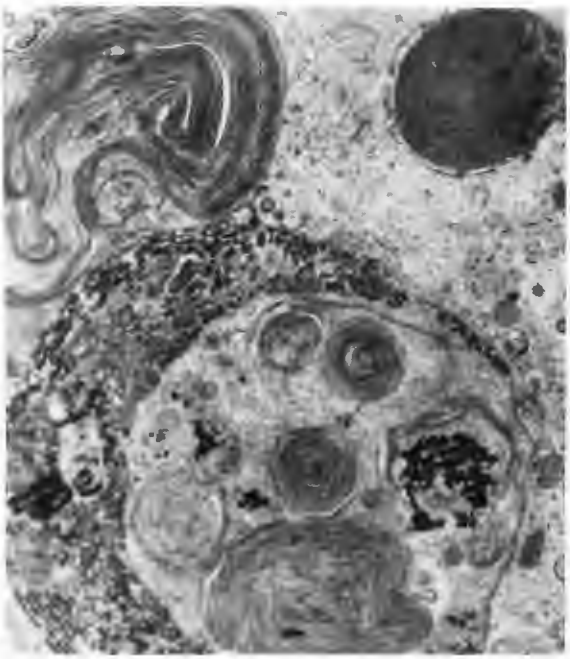


Figure 33. Induced 116 day midgut (x 14,000).



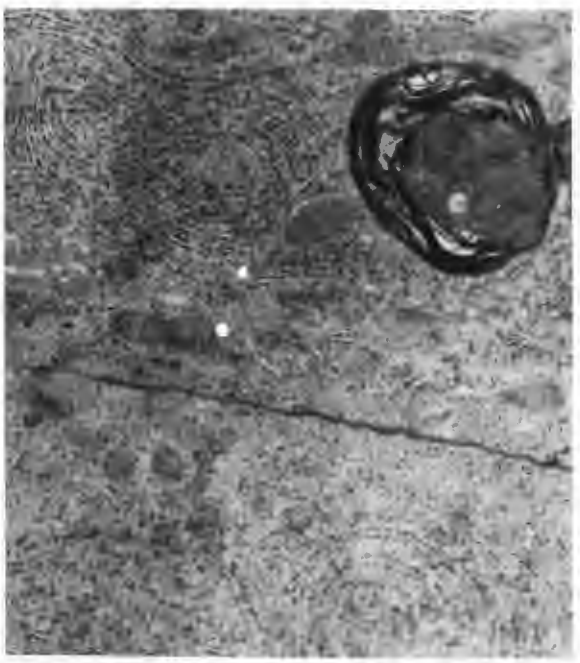


Figure 34. Midgut from a normal 130 day old female (x 12,000 and 16,500).

lamellar endoplasmic reticulum is again evident. The smooth profiles which were common 40 days earlier are now completely absent.

Figure 35 indicates the NADPH-NT-reductase activity in normal adult male American cockroaches. The enzyme activity is similar to that found in females except that it is considerably higher and the peak comes at a slightly later age. No other enzyme activities were measured in male insects.

Figure 36 shows the results of topical applications of carbaryl on mortality of male and female cockroaches of various ages. Dosages (100 mg/kg for females and 50 mg/kg for males) were adjusted to give approximate LD_{50} doses at 24 hours. Both males and females gave similar responses although the males were much more susceptible to the insecticide. Both sexes showed high susceptibility at young ages with increasing resistance up to an age of about 100 days. Beyond this age the animals quickly became more susceptible.

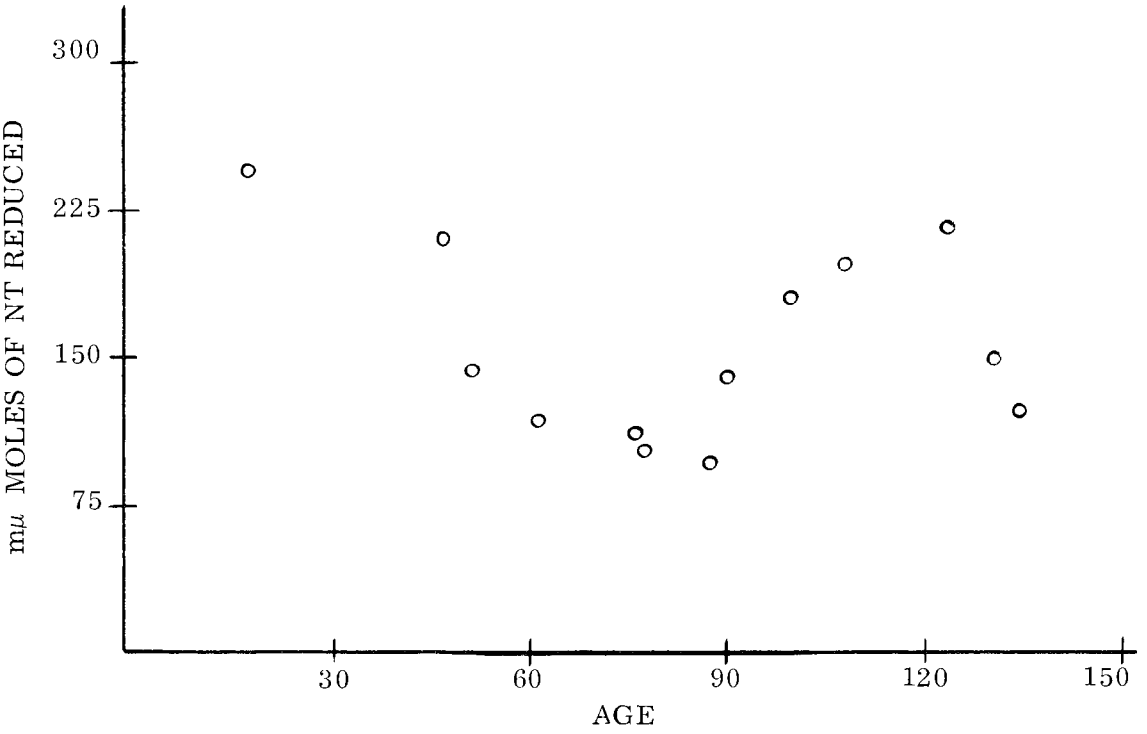


Figure 35. Levels of NADPH-NT-reductase activity per mg protein in normal adult male cockroach fat body.

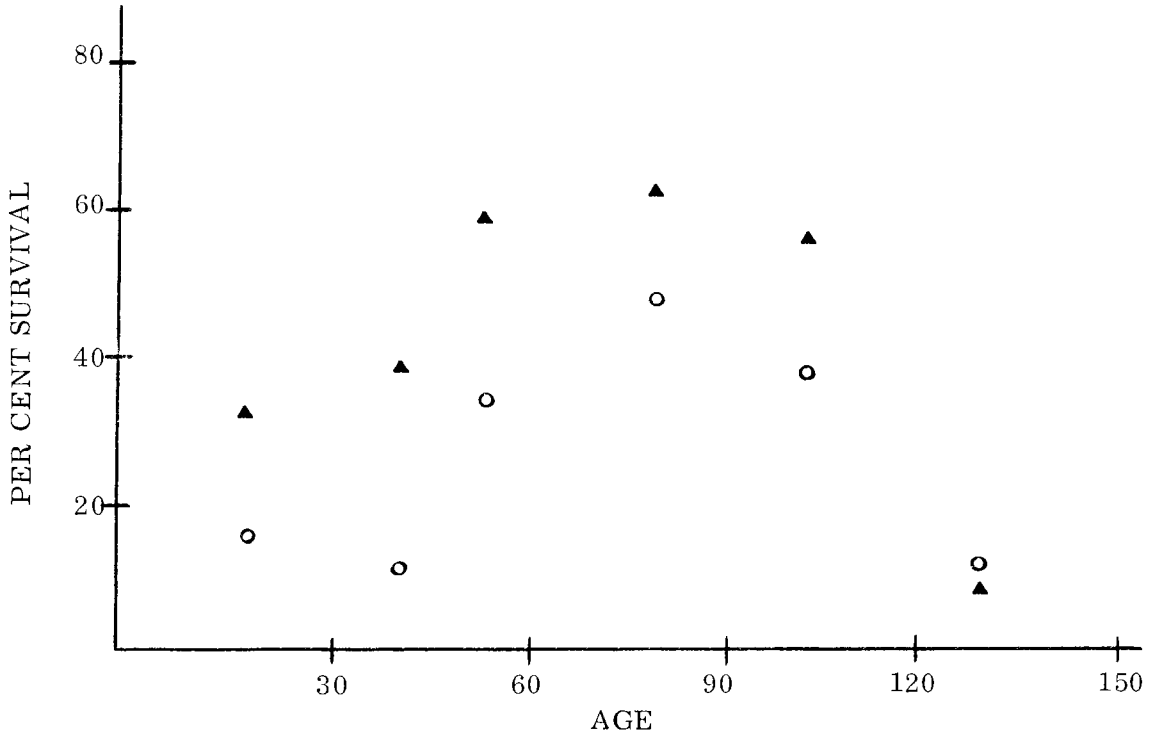


Figure 36. Twenty-four hour carbaryl toxicity in adult male (o) and female (▲) American cockroaches.

DISCUSSION

The American cockroach was chosen for studying the effects of age on insect microsomal mixed function oxidases for several reasons. It has a long adult life span, up to 180 days, which lends itself well to a study of this type. The insect is large which eliminates the need to use whole insect homogenates and instead allows the study of individual organs. There were also indications (Nakatsugawa and Dahm, 1965) that this insect showed definite, inherent, age-dependent changes in microsomal mixed function oxidase activities. Fat body, midgut, and hindgut tissues were chosen for study because of their size and accessibility and because these tissues are known to have relatively high microsomal mixed function oxidase activities (Nakatsugawa and Dahm, 1962).

The biochemical assays used were chosen in an attempt to measure all parts of the microsomal mixed function oxidase electron transport chain. The cytochrome P-450 determination is an actual measurement of the amount of the cytochrome present and not an activity determination. The NADPH-NT-reductase assay measures the activity of the flavoprotein component of the chain, the NADPH-cytochrome-c-reductase. The EPN-detoxication and p-nitroanisole O-demethylation assays indicate the activity of the chain as a whole. Thus, using these four assays, it was possible to determine the actual amount of one of the components of the chain, the activity of another, plus the activity of the entire chain.

It is evident from Figures 1 through 11 that the levels and activities of the microsomal mixed function oxidases vary greatly in American cockroach adults depending on the age of the insect. The findings that the activity increases up to about three months after the final moult is in close agreement with Nakatsugawa and Dahm's report (1965) that the insect showed an increasing ability to activate parathion to the same age. Parathion is known to be activated by the microsomal system.

The present study shows that cytochrome P-450 in fat body and EPN-detoxication and p-nitroanisole O-demethylation in fat body, midgut, and hindgut are very low in young adult insects. All rose steadily reaching a peak at about 90 days in midgut and hindgut and at about 100 days in fat body. The levels then fell rapidly during the next 30 to 40 days. It can be seen that the detoxication activities follow the levels of cytochrome P-450 quite closely. Some workers (Ichikawa et al., 1969; Lu et al., 1970) presented the idea that microsomal detoxication activity is a function of the ability of the electron transport chain to reduce the drug-cytochrome complex and is not dependent on the absolute amount of cytochrome P-450. This appears not to be the case in the American cockroach. The curves of cytochrome P-450 levels and detoxication rates are very nearly superimpossible. If xenobiotic detoxication were dependent on the reducing capacity of the chain it would be expected that this detoxication would mirror the NADPH-cytochrome-c-reductase activity since this flavoprotein is responsible for carrying electrons from NADPH to the cytochrome-drug complex. This was found not to be the case. NADPH-NT-reductase activity, which indicates the activity of the flavoprotein, was

elevated in young adult insects when detoxication activity was low. If detoxication were a function of the reducing capacity of the chain one would expect higher detoxication levels in young insects. Gram et al. (1968) reported that in rats p-nitroanisole O-demethylation followed cytochrome P-450 content quite closely. Remmer and Merker (1965) also reported that the rise and fall of cytochrome P-450 in induced rat liver parallels the change in drug-metabolizing activity. The question is certainly open to debate and needs further study.

The absolute amount of cytochrome P-450 compares closely with that found in house flies. Ray (1967) measured the quantity of the cytochrome in 3-4 day old house flies and, if protein concentrations are considered, these insects have about the amount of the cytochrome found in 50 to 70 day old American cockroaches.

The present study shows that in all three tissues there is an increasing ability to metabolize p-nitroanisole and EPN as the insects increase in age up to about three months, presumably because of increases in levels of the microsomal mixed function oxidase chain. After reaching this peak the enzyme levels fall off rapidly with a corresponding loss in xenobiotic detoxication ability.

On the basis of these results one would expect cockroaches of about three months of age to show a greater degree of resistance to toxic compounds than either younger or older animals since in vitro detoxication rates generally correspond to in vivo resistance. Figure 36 indicates this is true although resistance does not follow enzyme levels exactly. Fifty to sixty day old insects which have relatively low mixed function oxidase activities show a high level of

carbamate resistance. This does not necessarily mean that resistance is not due to increased carbaryl metabolism. Schonbrod and his coworkers (1968), using 14 strains of house flies, concluded there is no simple relationship between insecticide resistance and levels of microsomal oxidase activity. They suggested that more than one enzyme may be involved in the metabolism of different compounds. Also, in the present study, carbaryl metabolism was not measured *in vitro* and may show a different age-dependent metabolism curve than either *p*-nitroanisole or EPN. It might have been better if the toxic compound used was the same as the one used in the *in vitro* metabolism study. EPN was not used in the toxicity studies because it is an organophosphate which is activated by the same microsomal enzyme system which detoxifies it (O'Brien, 1959). In cases such as this it is difficult to determine if changes in toxicity are due to changes in activation, detoxication, or both (Dahm and Nakatsugawa, 1968).

Chlorcyclizine is known to act as an inducer of the microsomal mixed function oxidase electron transport chain. Wooles (1968) showed that injections of chlorcyclizine prevented ethanol-induced fatty liver in rats by stimulating hepatic microsomal activity. Welch and Coon (1963) reported that chlorcyclizine injections protected mice from organophosphate poisoning and Ahmad and Brindley (1969) showed that chlorcyclizine fed orally protected wax moth larvae against parathion. The same authors (1971) found that levels of EPN-detoxication, *p*-nitroanisole *O*-demethylation, and NADPH-NT-reductase activity were increased in wax moth larvae with injections of the drug. In the present study chlorcyclizine significantly increased levels and activities of the

microsomal mixed function oxidase chain in the American cockroach. The injections were effective only when normal enzyme levels were increasing, that is, when the insects were less than 100 days old. When normal enzyme levels were falling, between 100 and 130 days of age, the drug had no inductive effect.

RNA levels in normal midgut and hindgut were found to follow an age-dependent pattern similar to that of the microsomal electron transport chain. A peak was found at 90 days with a rapid decline after that point. The large increase in RNA levels from young to 90 day insects indicates that more than just the microsomal transport chain was being increased as the animals age. It would seem to indicate a general increase in overall metabolism in the tissue. The increase of the microsomal enzymes alone, only a small part of the complement of tissue enzymes, would not warrant the large RNA increases found.

Chlorcyclizine injections had very little effect on total RNA levels in midgut and hindgut tissues even though microsomal enzyme levels in these tissues were raised significantly. The reason for this is perhaps that only these enzymes were increased in the inductive process. Orrenius and Ericsson (1966b) reported that induction of rat liver increased levels of the microsomal oxidases but levels of glucose-6-phosphatase, ATPase, and NADH-cytochrome-c-reductase actually decreased. Remmer (1967) also reported that injections of phenobarbital caused increases of the microsomal oxidative enzymes of rats but that other hepatic enzymes did not increase. Considering the total amount of RNA in the tissue, the increased amount necessary to

elevate the levels of only the microsomal enzymes in induction might be too small to detect.

There is some disagreement as to whether enzyme induction is the result of increased protein synthesis or of decreased protein degradation. One bit of evidence in the present study seems to favor the theory of decreased degradation. Actinomycin D is known to be an inhibitor of RNA transcription from DNA (Schwartz and Garfalo, 1967). If induction is the result of increased synthesis of new protein an inhibitor such as this should block it. Some authors have indicated that protein inhibitors do indeed inhibit induction (Conney, 1967; Alvares et al., 1968) and that induction is preceded by synthesis of messenger RNA (Orrenius, 1965a). In the cockroach, however, actinomycin D did not inhibit the effects of the inducer but instead caused increases in the levels of the mixed function oxidases above and beyond those produced by the chlorcyclizine. These results are at best difficult to explain, and certainly more difficult if one assumes that induction is the result of increased enzyme synthesis. If decreased protein degradation is the mechanism of induction as some workers have postulated (Kuriyama et al., 1969) the results may be more plausable. Degradation of the microsomal proteins might involve specific catabolic enzymes which would be synthesized as other proteins with the production of messenger RNA. Drug induction might somehow interfere with the production of these degradative enzymes and so cause increased levels of the mixed function oxidases. Actinomycin D might further interfere with the production of these enzymes by inhibiting messenger RNA production and so result in even higher levels of the microsomal detoxifying system. Admittedly this mechanism does

seem somewhat contrived and it is difficult to see how the actinomycin D could inhibit the production of the catabolic enzymes without interfering with the production of the mixed function oxidases which must be occurring continually even if induction is the result of decreased degradation. The results however, while being very surprising and difficult to explain, are quite consistant, and certainly warrant further study.

It may seem contradictory that male cockroaches had a higher activity of at least one of the microsomal mixed function oxidase enzymes and yet showed a much lower resistance to topical applications of carbaryl. However, if one considers the relative amount of detoxifying tissue found in each sex the results are more easily explained. The insect fat body has long been considered the homologue of the mammalian liver as the main organ for the detoxication of foreign compounds. Female cockroaches have a great deal of fat body tissue (approximately 80 mg) while the same tissue is very sparse in the male insect (about 35 mg). Thus the male, even though having a higher enzyme activity per milligram of tissue, has lower overall detoxifying ability because of the reduced amounts of detoxifying tissue.

The term "microsome" is a somewhat artificial designation. It is not a true cellular entity but rather is classically defined as "the high speed pellet resulting when the supernatant fluid from the mitochondrial fraction is sedimented" (Siekevitz, 1965). It is considered to be composed primarily of fragments of smooth and rough endoplasmic reticulum. For this reason cytological studies of microsomal enzymes usually concern themselves with the structure

and distribution of the endoplasmic reticulum. An excellent review has been written by Dallner and Ernster (1968) concerning the composition of these subcellular structures.

Many workers have described the changes occurring inthe endoplasmic reticulum during the process of induction in mammals (Orrenius et al., 1965; Remmer and Merker, 1965: Staubli et al., 1969). Relatively little morphological correlation has been done with enzyme induction in insects and no one, to my knowledge, has studied morphological changes associated with normal mixed function oxidase level changes.

Electron micrographs of tissue from uninduced cockroaches 30 days old show "typical" rough endoplasmic reticulum. The membranes lie in parallel array or lamellae and the outer surfaces are covered with ribosomes. The cisternae of the membranes are very narrow. Changes can be noted in the appearance of the reticulum as the insects age and as microsomal enzyme levels increase. The cisternae become much more swollen and irregular in shape and some small vesicles can be seen. The fat body from uninduced 100 day old female cockroaches contains endoplasmic reticulum which is very similar to that found in rat hepatic tissue after receiving two injections of phenobarbital. Orrenius and Ericsson (1966a) have described this tissue as having endoplasmic reticulum which is greatly distended with the cisternae being filled with granular or flocculent material. They also reported that in some cells the rough reticulum seemed to be breaking up into vesicles. This trend can be seen in fat body from induced insects 100 days old. It is interesting that the early stages in this development can be seen in normal insects.

If mammals are subjected to repeated injections of inducing drugs the development of the endoplasmic reticulum leads to cells packed with smooth vesicles (Staubli et al., 1969). In the present study only a single injection of chlorcyclizine was administered so one might not expect smooth vesicles to be present. However, fat body from induced insects 100 days old show at least a few smooth profiles. In midgut, injections of all insects 90 days old or less resulted in the production of large amounts of smooth reticulum. In fact, even uninduced 90 day insects contain midgut which have many smooth tubules.

On the basis of the above results it would seem that the levels of microsomal mixed function oxidase activity can be estimated by noting the structure of the tissue's endoplasmic reticulum. Fat body with low enzymatic activity, either from normal 30 or normal 130 day old insects shows typical rough lamellar endoplasmic reticulum. Tissue from 30 day old induced insects and from uninduced 80 day old insects both show intermediate levels of enzymatic detoxication activity. Both tissues also show very similar endoplasmic reticulum morphology. The reticulum is rough in both cases but the lamellar appearance is no longer as evident. Single elements are frequently seen and some rough vesicular profiles are present. The same comparison can be made between fat body from 80 day induced insects and normal 100 day old insects. Both have high detoxication activities and both have endoplasmic reticulum which is rough, very irregular, and semi-vesicular in nature. Fat body from 100 day induced female cockroaches which showed the highest detoxication activity of any tissue examined, had endoplasmic reticulum which is becoming more tubular in nature.

Many of the vesicles are smooth or semi-smooth and even some of the long strands appear to be losing some of their ribosomes. The tissue is very similar to rat hepatic tissue after four days of injections of phenobarbital (Orrenius and Ericsson, 1966a). This tissue was reported to contain tubular and vesicular elements which had the dimensions of smooth endoplasmic reticulum but were coated or partially coated with ribosomes.

Comparisons can also be made between midgut tissues which show similar levels of enzymatic detoxication ability. Midgut of 30 and 130 day old uninduced cockroaches both show rough lamellar endoplasmic reticulum and both have similar low levels of detoxication activity. Midgut from 30 day old induced cockroaches and 90 day normal insects have intermediate levels of microsomal activity. The micrographs of the two tissues are nearly identical. Rough and smooth endoplasmic reticulum are present in about equal amounts. The rough reticulum is found as short single strands while the smooth vesicles are long and somewhat tubular. Tissue from 80 day induced insects, which is at the peak of midgut enzyme activity, is almost devoid of rough reticulum. Many smooth profiles are present and in some areas tightly pack the cell. This type of configuration was found in rat hepatic tissue only after five or six days of phenobarbital injections (Orrenius and Ericsson, 1966a).

From the cytological evidence it seems probable that there is a relationship between the morphological forms of the endoplasmic reticulum in induced and normal insects. Tissue from young animals can be made, with an injection of chlorcyclizine, to appear morphologically and have the same enzyme activity as tissue from a much older animal. The changes in the animal take place in

the 24 hours from the time of the injection instead of the many weeks which is required in normal development. Perhaps induction is simply a very accelerated form of normal cell development. Orrenius and Ericsson (1966a) hinted at this when they theorized that the change from rough to smooth endoplasmic reticulum in induction might be the natural way that smooth reticulum is produced but at a slower rate. It has been established that new membrane is formed from lipid and protein in the rough reticulum and transferred to the smooth surfaced part of the system (Dallner et al., 1966). Perhaps a long term "inducer" such as a hormone exerts an effect in normal development like chlorcyclizine does in induction.

Once the enzyme levels reach a peak at about 90 to 100 days of age they drop very rapidly. The ultrastructure gives a hint as to the mechanism of this rapid decline. Figure 30 shows midgut from 90 day induced female cockroaches. This tissue is from animals whose microsomal mixed function oxidase activity is just beginning to fall. It can be seen that very small membrane whorls are present in the lumen of some of the smooth vesicles. Figure 32 of the same tissue shows that acid phosphatase activity is also found in the lumen of the smooth endoplasmic riticulum. Although the small membrane whorls are not evident in this figure, the inference from Figures 30 and 32 is that the small membrane whorls are in the immediate area of high catabolic or cytolytic activity.

Figures 26 and 31 are from 105 day old normal and induced cockroaches respectively. A very dramatic difference is noted between this tissue and that

shown in Figures 25 and 30 which is of tissue only 15 days younger. The large areas of smooth endoplasmic reticulum are completely absent. Rough reticulum is found only as a few single elements scattered randomly throughout the cytoplasm. The membrane whorls are present but are much larger than those in 90 day old animals. It is evident that the chlorcyclizine injections had very little effect on the tissue in Figure 31. It appears to be almost exactly like that of the uninduced tissue of the same age in Figure 26. The enzyme activities of the two tissues are very nearly equal. It would seem that as the whorls increase in size the endoplasmic reticulum tends to disappear and the enzyme activities go down.

Figure 27 shows midgut from cockroaches 116 days of age. Large membrane whorls are present which show a positive acid phosphatase activity. It is evident that the membrane whorls or at least some cytoplasmic inclusions found within them are undergoing catabolic degradation. Older tissue shows that the whorls become very large and complex but that the rest of the tissue has come to look very much like that found in very young insects. It would seem that the tissue's physiology has been redirected from that of high enzymatic activity found in 90-100 day old insects to that of much lower activity found in 140 day old animals. The membrane whorls, by association, would seem to be implicated in this cellular reorientation. It would also seem that the simultaneous appearance of the membrane whorls and the disappearance of the large amounts of smooth endoplasmic reticulum may have something in common.

Membrane whorls were first observed by Palay and Palade (1955) who were studying the fine structure of nerve cells. They described the whorls

as onion-like corpuscles and assumed them to be derived from the endoplasmic reticulum. Emmelot and Benedetti (1960) injected rats with dimethylnitrosamine and found large membrane whorls in the liver. These whorls were seen to be continuous with smooth endoplasmic reticular membranes. Tooze and Davies (1965) showed that the structures were involved in the degradation of organelles during the normal maturation of erythrocytes. They showed the whorls to have acid phosphatase activity. Several workers have implicated the formation of the whorls with changing cell physiology. Ashford and Porter (1962) described them as being set aside for hydrolysis with the general purpose of providing the protoplast with breakdown products for use in reorientated physiology. Napalitano (1963) describes their formation as either "a prelude to cytolysis or as a result of acute reorientation of normal metabolic processes within the cell." Locke and Collins (1965) found the whorls in the fat body of insects and concluded the structures "undoubtedly serve to remove and condense cellular machinery no longer needed." Hruban et al. (1963) indicated that the whorls were important for the disposal of organelles when the cell changes its functional state and for the reutilization of materials.

Thus it seems likely that the membrane whorls found in the cockroach fat body and midgut are the result of the reorientation of the tissue's physiology. At least some of the reorientation involves the change from high to low microsomal detoxication ability. The large amounts of smooth endoplasmic reticulum likely are incorporated into the whorls beginning at about 90 days of age (Figure 30) in midgut and probably about 10 days later in the fat body. The whorls probably enlarge and combine to form the large structures seen in older animals

(Figure 34). While the whorls show acid phosphatase activity it is unlikely that the membranes themselves are subject to degradation. If they were it is unlikely that they could survive from the time of their formation until they were found in 140 day old insects. It seems more likely that the phosphatase activity is centered on cytoplasmic inclusions which have become trapped within the membranes.

The appearance of the whorls and the subsequent disappearance of the smooth endoplasmic reticulum offers a reason for the uninducibility of tissue after normal enzyme levels have begun to fall. Many workers have shown that the production of detoxifying enzymes in induction is dependent on the production of new membrane (Remmer and Merker, 1965; Orrenius and Ericsson, 1966a; Weibel et al., 1969). The smooth endoplasmic reticulum produced in induction is thought to be proliferated from existing rough reticulum (Orrenius et al., 1965). In cockroach tissue between 100 and 125 days of age almost all of the endoplasmic reticulum has disappeared, presumably having been incorporated into the membrane whorls. The tissue is uninducible simply because there is not enough endoplasmic reticulum present (Figure 29) to synthesize new enzyme. It is as if the cell is retooling or reorientating from having high microsomal detoxication ability to having the low activity found in 140 day old insects.

The present study gives no indication as to the reason for the steady increase in the microsomal enzyme levels in young insects or for the sudden fall of enzymatic activity in insects between 100 and 130 days of age. No correlation could be found with any physiological changes known to be inherent to the insect.

The ovarian cycle of the insect is 8-15 days and the females continue to lay viable egg capsules long after the enzyme peak has passed. Since the insects may live as long as two years, senility effects would not seem to be responsible for the falling enzyme levels after the peak is reached. Perhaps the enzyme levels are under hormonal control as suggested by Krieger and Wilkinson (1969) with a hormone acting as a long term inducer which produces an effect similar to chlorcyclizine but acting over a longer period of time. The production of the hormone might stop at about 100 days causing the "induction" to stop and result in a lowering of enzyme activity.

SUMMARY AND CONCLUSIONS

Microsomal mixed function oxidase enzyme activites in the American cockroach vary greatly with the age of the insect. The changes in levels of activity of this detoxication chain were accompanied by changes in susceptibility to carbaryl and by changes in the ultrastructure of the tissue. Detoxication activities in this insect were found to closely parallel the cytochrome P-450 content of the tissue rather than the reductive capacity of the electron transport chain.

Injections of chlorcyclizine were found to increase levels and activities of the microsomal detoxifying system only when normal enzyme levels were increasing. When basal levels were falling, after about 100 days of age, the tissues were essentially uninducible.

Tissue at the peak of enzyme activity, whether normal or induced, contained endoplasmic reticulum which was largely smooth in nature. As enzyme levels began to fall the endoplasmic reticular membranes disappeared and large concentric membrane whorls took their place. It seems likely that the membranes became incorporated into the whorls which showed positive acid phosphatase activity indicating a lysosome-like nature. It also seems likely that the absence of the reticular membranes in tissue whose enzyme activities were declining provides a reason for the uninducibility of such tissue since induction is generally thought to require these membranes.

Actinomycin D, an RNA synthesis inhibitor, was found to increase the effects of drug injections. These surprising results certainly warrant further study since the same treatment is known to block enzyme induction in mammals.

The present research indicates that when studying mixed function oxidase levels in normal or induced animals care must be taken to determine the age of the animal. Animals of different ages may show large differences in basal enzyme activities and in their response to inductive drugs. The research also indicates there may be a close relationship between normal enzyme increases and those found in enzyme induction since both are accompanied by similar morphological changes.

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APPENDIX

Table 2. Cytochrome P-450 levels in normal adult female cockroach fat body

Age	OD (450-490)[a]	Mg protein/ml	OD/mg protein
10	0.002	1.08	0.002
12	0.004	0.97	0.004
12	0.006	1.15	0.005
32	0.020	1.02	0.020
44	0.027	1.12	0.024
46	0.026	1.06	0.025
49	0.026	0.91	0.029
61	0.023	0.85	0.027
63	0.034	1.10	0.031
68	0.029	1.02	0.028
73	0.033	1.01	0.033
75	0.045	0.86	0.052
76	0.065	1.10	0.059
82	0.055	0.96	0.057
92	0.075	1.11	0.068
100	0.077	1.04	0.074
106	0.074	1.02	0.073
107	0.072	1.05	0.069
118	0.063	1.07	0.059
125	0.046	1.03	0.045
130	0.041	0.95	0.043

Table 2 (continued)

Age	OD (450-490)[a]	Mg protein/ml	Od/mg protein
142	0.021	1.00	0.021
149	0.011	1.10	0.010

[a]Levels are expressed as the optical density difference between 450 nm and 490 nm in solutions containing 10 mg of wet microsomes/ml.

Table 3. Cytochrome P-450 levels in induced adult female cockroach fat body

Age	OD (450-490)[a]	Mg protein/ml	OD/mg protein
8	0.044	1.21	0.036
40	0.056	1.24	0.045
53	0.066	1.14	0.058
56	0.076	1.19	0.064
71	0.085	1.10	0.077
82	0.093	1.12	0.083
91	0.100	1.03	0.103
99	0.104	1.12	0.093
107	0.084	1.05	0.080
119	0.054	0.98	0.055
132	0.047	1.06	0.044
145	0.018	0.94	0.017

[a]Levels are expressed as the optical density difference betwen 450 nm and 490 nm in solutions containing 10 mg of wet microsomal tissue/ml.

Table 4. NADPH-NT-reductase activity in normal adult female cockroach fat body

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
2	195	1.21	161
6	193	1.20	162
8	190	1.16	164
13	189	1.22	155
17	185	1.10	169
20	162	1.05	154
23	150	1.03	150
24	150	0.98	154
28	136	0.87	156
30	140	0.89	156
33	136	0.89	152
37	133	0.89	150
41	130	0.91	142
46	132	1.07	123
46	124	--	--
49	114	0.98	116
50	120	--	--
50	116	0.97	120
53	116	1.10	105
56	110	1.06	104

Table 4 (Continued)

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
57	104	1.04	100
59	106	--	--
62	110	1.11	100
63	112	1.14	98
65	106	1.04	102
67	120	1.10	109
69	105	0.92	115
73	138	1.05	132
74	140	1.00	140
79	146	0.98	149
83	150	0.98	153
88	164	1.05	156
90	166	1.00	166
92	168	1.04	161
93	171	1.05	163
94	168	1.05	160
96	171	1.15	149
98	170	1.10	155
103	166	1.25	135
107	156	1.24	126

Table 4 (Continued)

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
114	102	1.02	100
118	122	1.05	115
119	89	1.01	88
133	82	0.95	86
140	80	0.85	94
153	80	0.79	100

[a]Activity is expressed as mu moles of reduced neotetrazolium produced per ml per 10 minutes.

Table 5. NADPH-NT-reductase activity in induced adult female cockroach fat body

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
6	200	1.18	170
20	190	1.08	175
33	184	0.97	190
39	190	1.01	188
53	204	1.07	190
56	220	1.19	185
65	233	1.26	185
74	280	1.40	201
81	300	1.35	222
83	306	1.18	260
91	320	1.19	270
93	310	1.13	274
97	289	1.12	257
98	280	1.13	248
100	260	1.11	234
103	246	1.03	252
106	288	1.26	228
108	222	1.26	177
109	184	0.89	207
114	102	1.02	100
118	1.29	1.14	113
119	1.08	1.15	94

[a]Activity is expressed as mu moles of reduced neotetrazolium produced per ml per 10 minutes.

Table 6. EPN-detoxication in normal adult female cockroach fat body

Age	EPN-detoxication[a]	Mg protein/ml	EPN-detoxication/ mg protein
7	0.023	1.15	0.020
22	0.050	1.47	0.034
28	0.104	1.27	0.082
31	0.118	1.23	0.096
42	0.120	1.50	0.080
52	0.140	1.27	0.110
62	0.164	1.49	0.110
67	0.160	1.34	0.120
71	0.182	1.49	0.122
72	0.200	1.67	0.120
78	0.220	1.59	0.138
81	0.262	1.44	0.182
88	0.302	1.42	0.212
90	0.312	1.54	0.202
97	0.330	1.34	0.246
102	0.340	1.38	0.246
103	0.324	1.25	0.260
109	0.321	1.27	0.252
114	0.304	1.37	0.222
116	0.232	1.68	0.138
118	0.210	1.64	0.128
126	0.200	1.66	0.120
133	0.206	1.81	0.114

[a]Activity is expressed as ug of p-nitrophenol produced per 10 mg of wet microsomal tissue per hour.

Table 7. EPN-detoxication in induced adult female cockroach fat body

Age	EPN-detoxication[a]	Mg protein/ml	EPN-detoxication/ mg protein
10	0.136	1.66	0.082
22	0.176	1.49	0.118
42	0.224	1.25	0.180
60	0.276	1.52	0.182
75	0.334	1.04	0.320
81	0.380	0.99	0.382
90	0.400	1.24	0.332
95	0.470	1.18	0.398
97	0.476	1.25	0.380
101	0.460	1.15	0.402
103	0.456	1.16	0.394
103	0.398	1.10	0.362
104	0.440	1.37	0.322
109	0.446	1.57	0.284
115	0.300	1.49	0.202
118	0.240	1.43	0.168
129	0.234	1.50	0.156
131	0.222	1.71	0.130

[a]Activity is expressed as ug of p-nitrophenol produced per 10 mg of wet microsomal tissue per hour

Table 8. Activity of p-nitroanisole O-demethylation in normal adult female cockroach fat body

Age	p-nitroanisole O-demethylation[a]	Mg protein/ml	p-nitroanisole O-demethylation/ mg protein
7	0.028	1.15	0.024
22	0.088	1.47	0.060
28	0.140	1.27	0.110
31	0.155	1.23	0.126
42	0.198	1.50	0.132
51	0.208	1.39	0.150
62	0.214	1.49	0.144
67	0.201	1.34	0.150
78	0.344	1.59	0.216
88	0.332	1.42	0.234
90	0.442	1.54	0.286
102	0.384	1.38	0.278
103	0.353	1.25	0.282
109	0.370	1.27	0.292
114	0.249	1.37	0.182
120	0.215	1.04	0.206
131	0.202	1.10	0.182

[a]Activity is expressed as ug of p-nitrophenol produced per 10 mg of wet microsomal tissue per hour.

Table 9. Activity of p-nitroanisole O-demethylation in induced adult female cockroach fat body

Age	p-nitroanisole O-demethylation[a]	Mg protein/ml	p-nitroanisole O-demethylation/ mg protein
10	0.216	1.66	0.130
22	0.241	1.49	0.162
42	0.250	1.25	0.200
60	0.301	1.52	0.198
75	0.319	1.04	0.306
81	0.368	0.99	0.372
90	0.590	1.24	0.474
100	0.562	1.17	0.480
101	0.495	1.15	0.430
103	0.430	1.16	0.370
103	0.433	1.10	0.394
104	0.590	1.37	0.430
109	0.744	1.57	0.473
115	0.545	1.49	0.366
117	0.350	1.23	0.286
122	0.314	1.31	0.240
131	0.318	1.71	0.186

[a]Activity is expressed as ug of p-nitrophenol produced per 10 mg of wet microsomal tissue per hour.

Table 10. NADPH-NT-reductase activity in normal adult female cockroach midgut

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
14	76	1.19	64
17	85	1.25	68
21	43	0.67	64
25	50	0.76	66
30	77	1.18	65
31	79	1.22	65
38	30	0.45	67
39	84	1.23	68
43	49	0.74	66
50	57	0.84	68
54	92	1.34	69
62	36	0.59	61
65	39	0.74	53
68	73	1.33	55
69	37	0.73	51
70	63	1.50	42
71	17	0.46	37
76	37	1.03	36
80	24	0.65	37
87	22	0.58	38
90	46	1.15	40
90	75	1.47	51
91	66	1.20	55
91	68	1.31	52
93	54	1.17	46
93	60	1.05	57
94	34	0.64	53
95	39	0.80	49
99	55	0.89	62
102	63	1.03	61
108	64	1.02	63
110	71	1.15	62
110	29	0.53	55
115	53	0.96	55
124	61	1.11	55
125	54	1.02	53

Table 10 (continued)

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
130	22	0.61	36
130	30	0.86	35

[a]Activity is expressed as mu moles of NT reduced per ml of midgut mitochondrial supernatant per 10 minutes.

Table 11. NADPH-NT-reductase activity in induced adult female cockroach midgut

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
17	103	1.35	76
23	97	1.29	75
44	24	0.32	76
52	97	1.35	72
61	69	0.98	70
63	107	1.47	73
73	89	1.32	68
84	121	1.61	75
85	107	1.23	77
85	38	0.50	75
91	95	1.18	81
91	98	1.24	79
95	49	0.61	80
99	62	0.79	79
99	109	1.34	81
100	43	0.60	71
102	73	0.99	74
102	25	0.37	67
109	88	1.45	61
111	68	0.97	70
114	83	1.37	61
125	48	0.85	57

[a]Activity is expressed as mu moles of NT reduced per ml of midgut mitochondrial supernatant per 10 minutes.

Table 12. Activity of EPN-detoxication in normal adult female cockroach midgut

Age	EPN-detoxication[a]	Mg protein/ml	EPN-detoxication/ mg protein
14	0.070	1.19	0.060
30	0.090	1.19	0.076
31	0.094	1.23	0.076
43	0.050	0.75	0.068
54	0.121	1.33	0.091
62	0.100	0.58	0.172
79	0.210	0.98	0.214
86	0.200	0.86	0.232
90	0.202	0.88	0.228
94	0.132	0.64	0.202
100	0.240	1.33	0.182
102	0.240	1.32	0.183
110	0.130	1.15	0.112
110	0.040	0.53	0.076
115	0.060	0.96	0.062
130	0.040	0.61	0.066
130	0.060	0.71	0.084

[a]Activity is expressed as ug of p-nitrophenol produced per ml of mitochondrial supernatant per hour.

Table 13. Activity of EPN-detoxication in induced adult female cockroach midgut

Age	EPN-detoxication[a]	Mg protein/ml	EPN-detoxication/ mg protein
17	0.178	1.34	0.128
40	0.190	1.16	0.164
45	0.153	0.91	0.168
63	0.260	0.99	0.263
72	0.197	0.75	0.262
91	0.305	1.21	0.252
91	0.255	1.24	0.206
94	0.178	0.96	0.186
100	0.141	1.12	0.126
100	0.131	1.38	0.095
114	0.118	1.08	0.109

[a]Activity is expressed as ug of p-nitrophenol produced per ml of mitochondrial supernatant per hour.

Table 14. Activity of p-nitroanisole O-demethylation in normal adult female cockroach midgut

Age	p-nitroanisole[a] O-demethylation	Mg protein/ml	p-nitroanisole O-demethylation/ mg protein
14	0.090	1.19	0.076
21	0.070	0.67	0.104
30	0.130	1.19	0.110
41	0.080	0.83	0.096
50	0.080	0.84	0.096
52	0.060	0.59	0.102
54	0.130	1.33	0.098
62	0.066	0.58	0.112
65	0.100	0.74	0.136
68	0.260	1.33	0.196
76	0.180	1.02	0.176
77	0.380	1.42	0.198
85	0.230	1.06	0.216
86	0.160	0.86	0.186
87	0.134	0.58	0.230
90	0.242	1.15	0.210
94	0.146	0.64	0.230
100	0.250	1.32	0.190
102	0.180	1.03	0.176
106	0.110	0.72	0.152
108	0.150	1.02	0.148
110	0.100	1.15	0.088
110	0.034	0.53	0.064
115	0.090	0.96	0.092
130	0.040	0.61	0.066
130	0.034	0.71	0.048

[a] Activity is expressed as ug of p-nitrophenol produced per ml of mitochondrial supernatant per hour.

Table 15. Activity of p-nitroanisole O-demethylation in induced adult female cockroach midgut

Age	p-nitroanisole[a] O-demethylation	Mg protein/ml	p-nitroanisole O-demethylation/ mg protein
17	0.209	1.34	0.156
21	0.194	1.35	0.144
27	0.209	1.29	0.162
41	0.145	0.77	0.189
45	0.190	0.91	0.210
52	0.184	0.77	0.240
58	0.324	1.31	0.247
63	0.262	0.99	0.264
72	0.202	0.75	0.267
73	0.210	0.76	0.277
78	0.167	0.61	0.272
82	0.241	1.13	0.213
84	0.230	1.24	0.185
85	0.231	1.23	0.188
87	0.189	0.82	0.231
91	0.221	1.21	0.183
91	0.261	1.24	0.211
95	0.091	0.61	0.150
97	0.122	0.81	0.150
100	0.171	1.12	0.143
100	0.195	1.38	0.141
114	0.158	1.08	0.147

[a]Activity is expressed as ug of p-nitrophenol produced per ml of mitochondrial supernatant per hour.

Table 16. NADPH-NT-reductase activity in normal adult female cockroach hindgut

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
14	60	0.88	69
20	53	0.77	68
30	42	0.64	66
50	37	0.80	55
54	54	1.05	53
65	48	0.77	63
76	57	0.85	67
79	56	0.67	84
90	58	0.68	85
90	67	0.81	82
97	58	0.76	76
102	54	0.86	63
115	43	0.77	55
130	31	0.79	39

[a]Activity is expressed as mu moles of NT reduced per ml of hingdut mitochondrial supernatnat per 10 minutes

Table 17. EPN-detoxication activity in normal adult female cockroach hindgut

Age	EPN-detoxication	Mg protein/ml	EPN-detoxication/ mg protein
14	0.044	0.88	0.050
20	0.050	0.77	0.065
39	0.070	0.87	0.080
54	0.090	1.05	0.086
65	0.076	0.77	0.098
76	0.102	0.85	0.120
79	0.090	0.67	0.135
90	0.138	0.81	0.170
102	0.150	0.86	0.174
115	0.082	0.77	0.106
130	0.040	0.79	0.052

[a] Activity is expressed as ug of p-nitrophenol produced per ml of mitochondrial supernatant per hour.

Table 18. Activity of p-nitroanisole O-demethylation in normal adult female cockroach hindgut

Age	p-nitroanisole[a] O-demethylation	Mg protein/ml	p-nitroanisole O-demethylation/ mg protein
14	0.067	0.88	0.076
20	0.071	0.77	0.092
39	0.087	0.87	0.100
54	0.103	1.05	0.098
65	0.080	0.77	0.104
76	0.112	0.85	0.132
79	0.096	0.67	0.144
90	0.152	0.81	0.188
102	0.148	0.86	0.172
115	0.100	0.77	0.130
130	0.079	0.79	0.100

[a]Activity is expressed as ug of p-nitrophenol produced per ml of mitochondrial supernatant per hour.

Table 19. RNA levels in normal adult female cockroach midgut

Age	RNA[a]	Mg protein/ml	RNA/mg protein
30	82	1.19	69
50	60	0.84	71
54	86	1.33	65
65	54	0.74	73
76	100	1.02	98
79	96	0.98	97
90	121	1.14	106
90	103	0.87	118
105	92	0.96	96
130	52	0.86	61

[a]Levels are expressed as ug of RNA per ml of midgut mitochondrial supernatant.

Table 20. RNA levels in normal adult female cockroach hindgut

Age	RNA[a]	Mg protein/ml	RNA/mg protein
20	46	0.77	60
30	42	0.64	66
50	51	0.73	70
65	58	0.77	75
76	68	0.85	80
79	56	0.67	84
90	69	0.81	85
102	56	0.86	65
115	41	0.78	52
130	31	0.79	39

[a]Levels are expressed as ug of RNA per ml of hindgut mitochondrial supernatant.

Table 21. NADPH-NT-reductase activity in normal adult male cockroach fat body

Age	NT reduced[a] (mu moles)	Mg protein/ml	NT reduced/ mg protein
20	274	1.12	246
47	226	1.04	216
53	136	0.92	148
61	148	1.19	124
75	124	1.02	122
76	114	0.96	118
89	140	1.20	116
90	144	1.00	144
101	210	1.14	184
107	204	0.97	210
122	196	0.87	224
128	168	1.02	166
135	128	0.93	138

[a]Activity is expressed as NT reduced per 10 mg of microsomal tissue per 10 minutes.

Table 22. Twenty-four hour carbaryl toxicity in male and female American cockroaches

Age	Sex	Total insects	Total mortality	Per cent mortality	Per cent surviving
18	Male	23	20	83	17
	Female	59	39	66	34
37	Male	88	79	90	10
	Female	82	42	60	40
55	Male	22	14	64	36
	Female	35	14	40	60
80	Male	54	28	52	48
	Female	56	21	37	63
102	Male	48	29	60	40
	Female	31	13	42	58
130	Male	42	37	88	12
	Female	23	21	91	9

VITA

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Doctor of Philosophy

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